

# Detergent effects on disinfectant susceptibility of *Escherichia coli* and *Listeria monocytogenes* attached to stainless steel

by

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## Abstract

This study investigated the effect of detergent treatment on susceptibility of attached *Escherichia coli* and *Listeria monocytogenes* to subsequent disinfectant treatment, in relation to food industry cleaning procedures. *E. coli* attached to stainless steel surfaces became significantly more susceptible to benzalkonium chloride (BAC) after treatment with sodium alkyl sulphate (SAS) by 0.51 Log<sub>10</sub> cfu ml<sup>-1</sup> and fatty alcohol ethoxylate (FAE) by 0.96 Log<sub>10</sub> cfu ml<sup>-1</sup>. No change in susceptibility was observed with sodium dodecyl sulphate (SDS), sodium lauryl ethyl sulphate (SLES) or polyethoxylated alcohol (PEA). *L. monocytogenes* became significantly less susceptible to BAC after treatment with anionic detergents SAS by 0.79 Log<sub>10</sub> cfu ml<sup>-1</sup>, SDS by 0.33 Log<sub>10</sub> cfu ml<sup>-1</sup> and SLES by 0.22 Log<sub>10</sub> cfu ml<sup>-1</sup>, yet no change in susceptibility was observed with FAE.

Following treatment with all detergents both organisms became significantly more susceptible to sodium dichloroisocyanurate (NaDCC) demonstrating that the effect of the disinfectant was independent of detergent type.

Flow cytometry using the fluorochrome propidium iodide (PI) revealed significant increases in cell membrane permeability of both organisms by all detergents except sodium dodecyl sulphate (SDS) and the effect was much greater in *E. coli*. Increasing above the in-use concentration of SAS and FAE had no further effect on cell membrane permeability, or susceptibility to BAC.

Hydrophobic interaction chromatography (HIC) showed that *E. coli* became less hydrophobic following treatment with SAS, SDS, FAE and

*L. monocytogenes* became less hydrophobic following treatment with SAS and SDS but no effect was seen with FAE.

Investigations into carbon chain length of detergent revealed that SAS and the C18 standard increased susceptibility of *E. coli* to BAC which, with permeability results, suggests a link between increase in susceptibility to BAC and increase in membrane permeability.

Efflux experiments with *L. monocytogenes* showed that efflux of ethidium bromide (EtBr) was greater from cells treated with SAS than with FAE

suggesting that the anionic charge on the detergent molecule influences an efflux mechanism that reduces susceptibility to BAC.

Overall the results demonstrate that detergent type can influence the sensitivity of persistent food borne microorganisms to BAC and NaDCC and the significance of the findings may impact on the choice of agents used in cleaning procedures in the food industry.

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## **ABBREVIATIONS**

<b>a.m.u.</b>	Atomic mass units
<b>ANOVA</b>	Analysis of variance
<b>BAC</b>	Benzalkonium chloride
<b>CCFRA</b>	Campden and Chorleywood Food Research Association
<b>CM</b>	Cytoplasmic membrane
<b>CSH</b>	Cell surface hydrophobicity
<b>CPZ</b>	Chlorpromazine Hydrochloride
<b>EPI</b>	Efflux pump inhibitor
<b>EPS</b>	Extra cellular polysaccharide
<b>EtBr</b>	Ethidium Bromide
<b>FAC</b>	Free available chlorine
<b>FAE</b>	Fatty alcohol ethoxylate
<b>FL3</b>	Fluorescence at 635 nm
<b>FS</b>	Forward scatter
<b>MS</b>	Mass spectrometry
<b>HIC</b>	Hydrophobic interaction chromatography
<b>HOCL</b>	Hypochlorous acid
<b>HT</b>	Hepes Tris
<b>LPS</b>	Lipopolysaccharide
<b>MATE</b>	Multidrug and toxic compound extrusion
<b>MDR</b>	Multidrug resistance
<b>MFS</b>	Major facilitator family
<b>MS</b>	Mass spectrometry

<b>PEA</b>	Polyethoxylated alcohol
<b>NaDCC</b>	Sodium dichloroisocyanurate
<b>NaOH</b>	Sodium hydroxide
<b>OM</b>	Outer membrane
<b>rpm</b>	revolution per minute
<b>PI</b>	Propidium Iodide
<b>QACs</b>	Quaternary ammonium compounds
<b>RND</b>	Resistance nodulation division
<b>SAS</b>	Sodium Alkyl Sulfate
<b>SDS</b>	Sodium dodecyl sulphate
<b>SDW</b>	Sterilised distilled water
<b>SE</b>	Standard error
<b>SLES</b>	Sodium lauryl ether sulphate
<b>SMR</b>	Small multidrug resistance
<b>SS</b>	Side scatter
<b>TSA</b>	Tryptone soya agar
<b>TSB</b>	Tryptone soya broth
<b>TVC</b>	Total viable count
<b>WOSH</b>	Water of standard hardness



**1.1 Food borne disease**

Food borne illnesses have been defined as infectious or toxic diseases, caused by agents that enter the body through the ingestion of food (WHO, 2012) which may have been cross-contaminated from the air, from food handlers or from surfaces during preparation (Kumar and Amand, 1998; Hood and Zottola, 1997; Rivas *et al.*, 2007). Contamination of food products leads to a negative impact on the storage, quality and safety of that food (Helke *et al.*, 1993; Hood and Zottola, 1995) and Troller (1993) estimated that 25% of food borne disease was caused by product contamination which is particularly unacceptable in ready-to-eat foods that are not subjected to further processing procedures. Infections due to food borne diseases caused by pathogenic strains of different organisms (Table 1.1) have emerged over recent decades and although their incidence is relatively low, their severe and sometimes fatal health consequences, particularly among infants and the elderly, make them serious food borne infections (WHO, 2012).

Table 1.1 Epidemiological data (Health Protection Agency, 2012)

Pathogen	Human cases in England and Wales reported to the HPA Centre for Infections in 2010
<i>Campylobacter</i>	62,684
<i>Escherichia coli</i> O157	793
<i>Escherichia coli</i>	27,055
<i>Salmonella</i>	9,071
<i>Staphylococcus aureus</i>	10,070
<i>Listeria monocytogenes</i>	156

### 1.1.1 *Escherichia coli*

*E. coli* is a Gram-negative, rod shaped bacterium that is commonly found in the gut of humans and other warm-blooded animals. In 2010, there were 27,055 reports of food borne disease for *E. coli* in the UK, which was a 5 % increase compared to 2009 and, since 2006, there has been a 35 % increase in *E. coli* bacteraemia reports (HPA, 2012). Although most strains are harmless, some such as Shiga toxin producing *E. coli* (STEC) can cause severe food-borne disease. There have been several notable outbreaks associated with STEC O157:H7 (Dundas *et al.*, 2001; Payne *et al.*, 2003), which is the most predominant serotype (Rivas *et al.*, 2007) that can lead to severe complications such as haemolytic uremic syndrome (HUS), which is the most common form of acute renal failure in children (Karmali, 1989). However, non O157 STEC infections have caused 10 – 30 % of HUS cases in the United Kingdom (Kleanthous *et al.*, 1990) and in several other countries around the world (Caprioli *et al.*, 1997). The infection is usually

transmitted through consumption of contaminated water or food and symptoms include abdominal cramps, diarrhoea, fever and vomiting. While most patients recover within 10 days, in some cases the disease becomes life threatening (WHO, 2012).

Gram-negative bacteria are intrinsically more resistant than Gram-positive to disinfectants such as quaternary ammonium compounds (QACs) (Langsrud *et al.*, 2004), which has been attributed to the relatively impermeable outer membrane (McDonnell and Russell, 1999).

### **1.1.2 *Listeria monocytogenes***

*L. monocytogenes* is a Gram-positive, rod shaped pathogenic bacterium that attaches and grows on surfaces even at low temperatures (Mafu, 1990, Wirtanen and Mattila-Sandholm, 1993; Heir *et al.*, 2004; Aarnisalo *et al.*, 2007). *L. monocytogenes* are ubiquitous in the environment, and have become a food-borne pathogen of great concern to the food industry (Briandet *et al.*, 1999; Fonnesbach *et al.*, 2001; Heir *et al.*, 2004; Soumet *et al.*, 2005; Gram *et al.*, 2006; Wilks *et al.*, 2006; Lourenco *et al.*, 2009) as it is commonly isolated from the dairy industry and food production sites (Aarnisalo *et al.*, 2007).

*L.monocytogenes* are able to grow at a wide range of temperature and pH, and as a psychrotrophic pathogen is able to grow at refrigerator temperatures and is hazardous with respect to chilled food products (Mustapha and Liewen, 1989; Kiss *et al.*, 2006; Wilks, 2006), with ready to eat foods particularly being considered a risk (Soumet *et al.*, 2005). As there is potential for growth in high risk foods during storage, the Health Protection

Agency (HPA) (2012) recommends reviews of the food preparation environment, including cleaning, for cases where  $10 \leq 10^2$  of *L. monocytogenes* are found in 25 g of food with satisfactory numbers being <10 organisms / 25 g.

It is of major concern to the food industry as *L. monocytogenes* is able to persist as a resident organism in food processing environments for many years (Unnerstad *et al.*, 1996; Bagge-Raven *et al.*, 2003; Fonnesbach *et al.*, 2001; Heir *et al.*, 2004) with some strains causing prolonged contamination (Rorvik *et al.*, 1995) and many outbreaks are linked to cross contamination from surfaces or equipment (Wilks *et al.*, 2006).

*L. monocytogenes* can lead to illness such as listeriosis, meningitis and septicaemia, which can often be fatal in high-risk groups such as pregnant women, neonates, the elderly and people with weakened immune systems (Best *et al.*, 1990; Soumet *et al.*, 2005; Kiss *et al.*, 2006; Thevenot *et al.*, 2006; HPA, 2012) and may also lead to public health problems and economical loss (Lourenco, 2009). During 2010, 156 human cases were reported to the HPA Centre for Infections in England and Wales (HPA, 2012), and although cases of food poisoning from *Listeria* are fewer than other pathogens (Table 1.1), listeriosis has a high fatality rate of 20 – 30% of cases (Godreuil *et al.*, 2003; Wilks, 2006).

Elimination of *L. monocytogenes* has been proved very difficult despite the implementation of regular cleaning and disinfection treatments (Aarnisalo *et al.*, 2007) and has been recovered from a variety of surfaces after normal

cleaning procedures (Romanova *et al.*, 2007). However, it is not known if its persistence in food processing environments is a result of poor cleaning and disinfection procedures or to adaptation or resistance to the products used (Lunden *et al.*, 2003; Gram *et al.*, 2006). Work carried out by Kiss *et al.* (2006), found that not only were *L. monocytogenes* strains present in food samples but also in samples taken from food production equipment and Gram *et al.* (2006) also demonstrated that the efficacy of cleaning and disinfecting products against *L. monocytogenes* is highly dependent on the food matrix in which the organisms are embedded.

### **1.1.3. Surfaces**

Bacteria have the ability to attach to different types of surface (Notermans *et al.*, 1991) and attachment is affected by the physiochemical surface properties of the surfaces and the microorganisms (Mafu *et al.*, 1991; Boulange-Petermann *et al.*, 1995). The hygiene of the food contact surface in a processing environment is crucial to the safety of the food that is passing along it during production. Stainless steel is the most commonly used material for the construction of food processing surfaces (Hood and Zottola, 1997; Frank, 2001) as it satisfies the requirements for the hygienic production of food of being chemically and physiologically stable at a wide range of production temperatures, durable, easy to clean and highly resistant to corrosion (Verran *et al.*, 2001). It does not absorb strong flavours and smells from foods, is non-tainting and does not release any harmful chemicals. It is able to withstand the effects of repeated cleaning with chemical cleaners and is corrosion resistant to acidic products such as tomato sauce. The most

commonly used grade is AISI 316, which is iron with 18% chromium and 9% nickel and is used throughout the production chain from manufacture to storage and food preparation in large-scale catering kitchens (Boulange – Petermann, 1996). Despite all of its advantages for use in the food production area, Wilks *et al.* (2006) observed that *E. coli* survived for longer periods of time on stainless steel surfaces, compared to other metals, emphasising the necessity for efficient hygiene procedures.

## **1.2 Attachment and biofilms**

Many bacteria are capable of attaching to surfaces (Frank and Koffi, 1990; Kryszinski *et al.*, 1992; Helke *et al.*, 1993) such as *Pseudomonas*, which are common spoilage organisms of perishable foods (Hood and Zottola, 1997), and pathogens such as *L. monocytogenes* which are widespread in the environment and are often found in food processing establishments (Mafu *et al.*, 1990; Kryszinski *et al.*, 1992). Attachment involves a series of stages as organisms such as *L. monocytogenes* move toward stainless steel surfaces (Ronner and Wong, 1993; Briandet *et al.*, 1999) through gravity, Brownian motion or motility by appendages that carry the organisms to a point where attachment can occur by fibrils or electrostatic interactions (Mustapha and Liewen, 1989; Boulange-Petermann 1996; Blackman and Frank, 1996). Allison and Matthews (1992) and Ganesh and Anand (1998) describe how adhesion to a submerged surface begins with the laying down of organic and inorganic molecules, such as proteins from milk and meat, that adsorb onto a surface.

Through the influence of the physiochemical properties of the bacterial surface, including surface charge and cell surface hydrophobicity (Van Loosdrecht *et al.*, 1987), forces act to promote an interaction to the conditioned surface before reversible adhesion occurs (Hood and Zottola 1997; Ganesh and Anand, 1998). Adhesion occurs through electrostatic and Van der Waals forces, which are weak interactions that operate over a range of approximately 10-20 nm and 50 nm respectively between the negative charges of an inert surface and the extra cellular polysaccharide (EPS) of the bacterial cell matrix (Boulangue-Petermann, 1996). Forces of repulsion are generated by the negative charges of the stainless steel surface and the cell surface that act to prevent the bacteria making contact with the surface but this is overcome by fimbriae that penetrate the energy barrier and allow short-range forces to operate. These are chemical bonding and hydrophobic interactions that are dependent on the ionic strength of the aqueous environment and operate at distances of greater than 1 nm. An increase in the ionic strength of the liquid medium increases the bacterial adhesion and adsorption by reducing the repulsive electrostatic interactions (Boulangue - Petermann, 1996).

The type of surface, the liquid environment and the microorganism are all factors that affect the adhesion process. Over a period of time the attachment of bacteria may involve synthesis of adhesions and EPS (Krysinski *et al.*, 1992; Ronner and Wong, 1993) that may bring about physiological changes in the organisms (Gram *et al.*, 2006). Boulangue - Petermann (1996) described EPS as macromolecules that comprise the peptidoglycan or the capsule, for Gram-positive species of bacteria, or

proteins and lipopolysaccharides for Gram-negative species of bacteria. Contrary to this, many authors (Notermans *et al.*, 1991; Hood and Zottola, 1997; Lindsay and von Holy, 1997) describe EPS as being produced by the cell and released to the outside of the cell wall where it forms a means of attachment and a matrix for protection. The outer layer formed around the bacteria, by the EPS, is usually polyanionic in nature (Gibson *et al.*, 1999; Underwood, 2004) and has hydrophilic and hydrophobic properties that determine how it reacts at the cell-surface interface (Allison, 1998).

It has been observed that bacteria grown on solid media produce more EPS than those cultivated in a liquid broth (Allison and Matthews, 1992) and Allison (1998) reported that in studies of *Pseudomonas aeruginosa*, a low molecular weight EPS was produced in response to the presence of a solid surface. Hood and Zottola (1997) also suggested that contact with a surface such as stainless steel might trigger the mechanism for the production of polysaccharides. Conditions in a food-processing environment favour attachment of organisms and biofilm formation can develop in a relatively short period of time depending on the particular environmental conditions (Holah, 1995; Gibson *et al.*, 1999).

After the initial attachment of the cell to the surface, more EPS is produced. More cells attach to the EPS, and layers of immobilised organisms are produced to form biofilms that are permeated by water channels that help trap nutrients (Hood and Zottola, 1997) and form a significant risk as the bacteria have the potential to come into contact with foods (Knight and Craven, 2010).



While bacteria can adhere to a surface in minutes, it is generally assumed that biofilms may take hours or days to form (Hood and Zottola, 1995). Single cells attached to surfaces are as important as well developed biofilms (Hood and Zottola, 1995) which have been widely investigated (Holah, 1995; Ganesh and Anand, 1998) as they form potential hazards to the food industry particularly through contamination from pathogenic bacteria, which occurs if viable cells desorb or are broken away by the physical movement of the product passing over the contaminated surface. Costerton (1995) suggested that the conversion from a planktonic cell to a biofilm causes distinct phenotypic changes that induce adhesion and influence the resistance of the biofilm bacteria to antibacterial agents. Several authors agree that bacteria adhered to surfaces, that form micro colonies, are more resistant to the adverse treatments of cleaning and disinfection products than bacteria in suspension (Frank and Koffi 1990; Dhir and Dodd, 1995; Boulange-Petermann, 1996; Gilbert *et al.*, 1998; Briandet *et al.*, 1999; Norwood and Gilmour, 2000; Gram *et al.*, 2006; Romanova *et al.*, 2007) and Mosteller and Bishop (1993) agreed the effect of the disinfectant can be reduced by biofilm growth. Mustapha and Liewen (1989), Sakagami *et al.* (1989), Frank and Koffi (1990) and Aarnisalo *et al.* (2007) all stated that this was due to the EPS providing protection against and reducing the efficacy of chemical sanitizers and Rivas *et al.* (2007) agreed that survival was enhanced in diverse environments.

### 1.3 Control of contamination

In the food industry, regular cleaning procedures are applied to reduce or eliminate spoilage and pathogenic microorganisms (Soumet *et al.*, 2005) such as *E. coli* and *L. monocytogenes* that are prevalent in foods and the environment and have the potential to cause serious illness (Wilks *et al.*, 2006) through the contamination of foods (Gram *et al.*, 2006). The cleaning procedure (Figure 1.1) involves the use of detergents that break down and remove food soils (Carpentier and Cerf, 1993) and most of the microorganisms (Cerf *et al.*, 2010). To reduce the viability of the remaining organisms, disinfectant is applied for 5 minutes at a temperature that is appropriate for the environment according to manufacturer's recommendations. Gibson *et al.* (1999) observed that while the use of detergent reduced the concentration of microorganisms, a disinfectant was still required as significant numbers remained on surfaces and Kuda *et al.* (2011) observed that disinfectant alone was unable to achieve that same level of sanitation on surfaces as those that were washed with water prior to disinfection. The effectiveness of the cleaning and disinfectant products differs depending on the target bacteria and type of soiling (Gram *et al.*, 2006). The efficacy of the cleaning procedure is crucial in reducing levels of organisms that may become tolerant to the cleaning procedures and in minimising the transfer of organisms from surface to product, where they have the potential to cause serious illness involving large numbers of people.

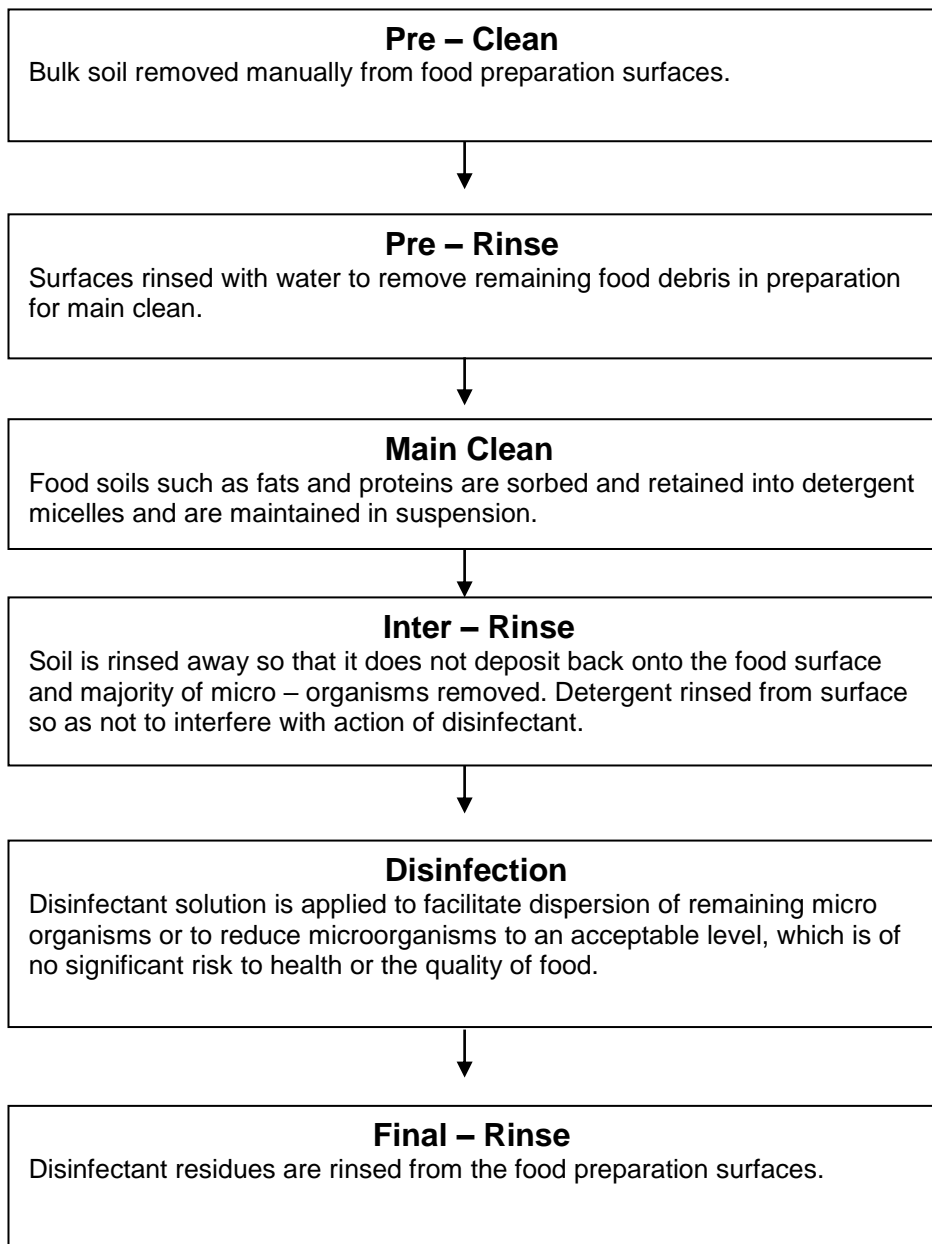


Figure 1.1. Food industry sanitation programme adapted from Holah (1995)

Some contact surfaces may typically be cleaned several times a day however; if the cleaning programme is not effective then microorganisms may not be destroyed (Gibson *et al.*, 1999) and sub lethally injured cells may recover to recontaminate surfaces and food. It would be expected that following the cleaning procedure, the total viable count (TVC) of microorganisms on a surface would be significantly reduced however, previous work by Hayes (2006) observed that some combinations of detergent and disinfectant did not cause a reduction in TVC following the cleaning process suggesting a decrease in susceptibility to the products used. On the other hand some organisms showed a greater than expected reduction in TVC suggesting an increase in susceptibility to the products used.

There has been much published on bacterial resistance of clinical strains to disinfectants (McDonnell and Russell, 1999; Russell, 2001), and several studies using food-associated bacteria such as *L. monocytogenes* and *Staphylococcus* spp have also observed resistance to quaternary ammonium compounds (QACs) (Heir *et al.*, 1995; Aase *et al.*, 2000; Langsrud *et al.*, 2003). However, a search of the literature has shown little work published on detergent / disinfectant combinations (Brown and Richards, 1964; Gram *et al.*, 2006) and none that has a direct relevance to the food industry.

### 1.3.1 Detergents

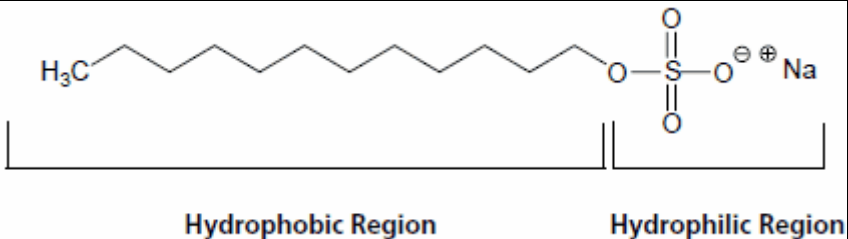
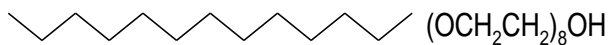
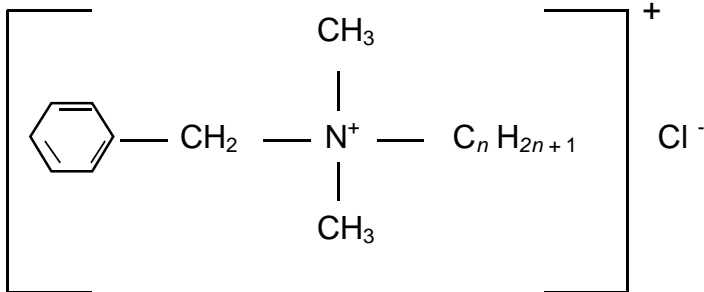
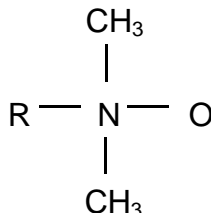
Following a period of production, food soil remaining on surfaces will generally consist of protein, fat and carbohydrate (Holah, 1995). There will also be microbial soil, which may be bacteria, attached for short periods of time, dried on cells or biofilms of organisms that have built up over a longer period of time. Effective methods for the control of pathogenic and food spoilage bacteria in food processing environments include the cleaning of food contact surfaces that involves the use of detergents that are rinsed away before the application disinfectant

Detergents are also known as surfactants, or surface-active agents, and chemically synthesised surfactants are commonly used in the food industry as emulsifiers or wetting agents as their properties enable them to lower the surface tensions of aqueous solutions and increase the solubility of insoluble compounds (Singer and Tjeerdema, 1993; Singh *et al.*, 2006). Chemically manufactured detergents used in the food industry contain different compounds, including surfactants (Morelli and Szajer, 2000) and are mixtures of alkyl chains (Lewis, 1995) with anionic detergents being used most frequently (Singer and Tjeerdema, 1993).

Detergents are not primarily intended to have any antimicrobial activity but are designed to break down food soils, such as protein substances, and remove most of the microorganisms present (Holah, 1995). The use of detergents is an important procedure before applying disinfectant as the presence of organic and inorganic soil can potentially act as protection to microorganisms or inactivate a disinfectant (Holah, 1995; Gibson *et al.*, 1999). However, it is also important that the detergent itself must be able to

be rinsed away without leaving a deposit on the surface (Underwood, 2004) as it may react chemically with the disinfectant and destroy its antimicrobial properties (Holah, 1995) or leave behind food soil residues that lower its effectiveness (Mustapha and Liewen, 1989). As a result their approved use for the food industry, to remove organic matter that would inactivate the disinfectant, means that low concentrations are used for sanitization that do not leave any toxic residues (Gardner and Peel, 2001). However, inadequate use of detergent in cleaning prior to disinfectant treatment can provide conditions suitable for the adaptation of sensitive bacteria (Aase *et al.*, 2000). Detergents are amphipathic molecules that contain both a hydrophilic (polar) 'head' group and a hydrophobic (non polar) hydrocarbon 'tail' with a chain length of C10 – C17 which can be linear, branched or aromatic (Singer and Tjeerdema, 1993). The hydrophilic head group is either an ionic or highly polar non-ionic group which solubilises in water (Brown, 2005) to become the active ion when the detergent dissociates. If the active ion is negatively charged the detergent is classified as anionic and if positively charged it is cationic. The head group of non-ionic surfactants usually consists of ethylene oxide units (ethoxylated compounds), which do not ionise in solution. The active ions from amphoteric detergents can be positively or negatively charged depending on the pH of the solution (Table 1.2).

Table 1.2. Types and properties of commonly used detergents used in the food industry

	Examples	Properties	Structure	References
Anionic	Sodium lauryl sulphate, Sodium alkyl ether sulphate, Sodium dodecyl sulphate.	Active ion is negatively charged. Can induce bacterial lysis. More active against Gram +ve bacteria than Gram -ve bacteria.		Im <i>et al.</i> , (2008)
Non - ionic	Alcohol ethoxylate. Polyethoxylated alcohol. Polysorbates.	Do not ionise in solution. Generally assumed not to be bactericidal but can cause damage to the cytoplasmic membrane.		Rangel-Yagui <i>et al.</i> , (2005)
Cationic	Benzalkonium chloride.	Active ion is positively charged. Bactericidal—alters permeability of cell membrane leading to loss of function and cell death.		McDonnell and Russell (1999)
Amphoteric	Dodecyl-diaminoethyl-glycine. Dodecyl-β-alanine	Active ions can be positively charged depending on the pH. Bactericidal when cationic at acidic pH.	 <p style="text-align: center;">R: C8 – C18</p>	Koike <i>et al.</i> , (2007)

Polar or hydrophilic substances dissolve in water because they are able to form hydrogen bonds and electrostatic interactions with water molecules so the polar head of the detergent molecule disrupts the hydrogen bonding and forms hydrogen bonds with water molecules. Non-polar or hydrophobic substances are unable to form such interactions and are immiscible with water (Bhairi and Mohan, 2007). This results in the hydrocarbon chains aggregating, due to hydrophobic interactions, to form spherical structures called micelles, which have hydrophobic cores.

This is fundamental in the soil removal process as the hydrophobic core region of the detergent micelle associates with the hydrophobic surfaces of proteins resulting in a soluble protein-detergent complexes that retains the soil in a suspension and is rinsed away following detergent treatment (Figure 1.2). Rinsing away of the detergent is important as anionic soaps and synthetic detergents, which carry opposite electrical charges, inactivate disinfectants and many non-ionic detergents are known to inactivate QACs (Gardner and Peel, 2001).



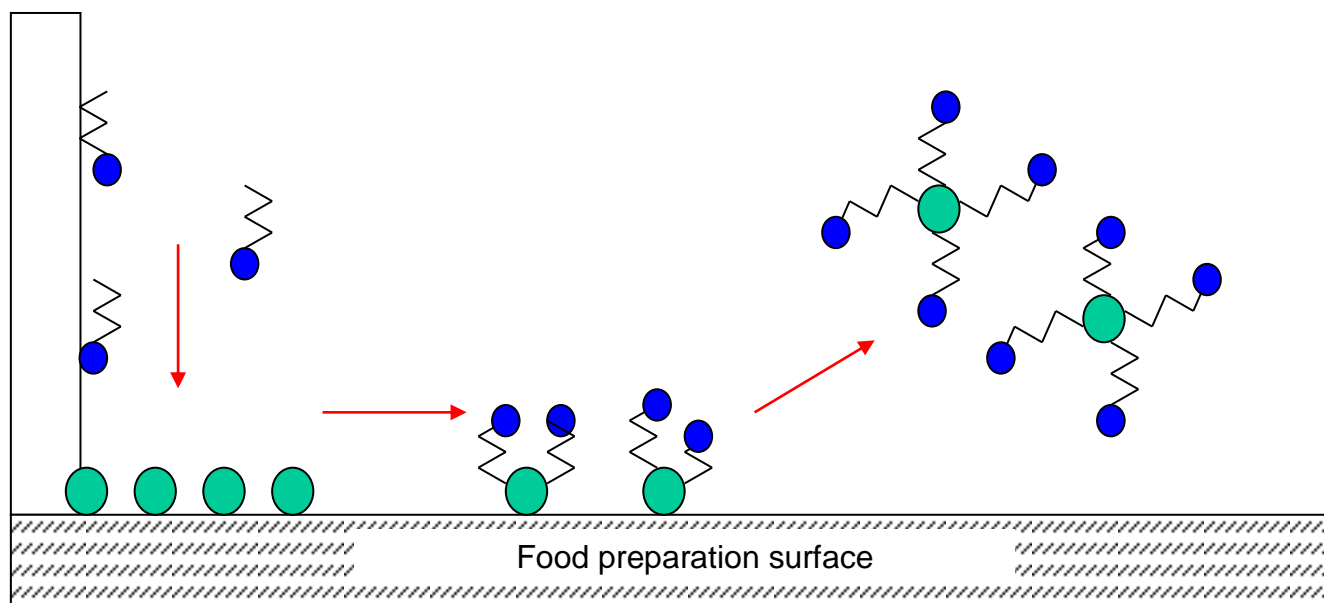


Figure 1.2. Hydrophobic interactions between detergent and food soil and aggregation of hydrophobic chains to form micelles. ● = detergent; ● = fatty food soil.

The differences observed in antimicrobial activity are dependent on the detergent molecule, its interactions with the cell envelope and passage to the cytoplasmic membrane. The outer membrane of the Gram-negative cell wall with its lipopolysaccharide (LPS) is considered a permeability barrier (Joynson *et al*, 2002) that protects the inner membrane against compounds toxic to the cell. Passage through this barrier would require disruption of electrostatic forces that stabilise the LPS and possible passage through the porin channels to access the inner membrane and cause structural damage. The Gram-positive cell wall does not offer this protection to the cytoplasmic membrane and in general Gram-positive organisms are more susceptible to antimicrobial agents.

Initially a detergent will interact with the surface of the cell and efficiency of cationic agents will be determined by the positively charged head group and alkyl chain length. For anionic detergents efficiency will be dependent on targeting and solubilising membrane- bound enzymes (Denyer and Stewart, 1998) and according to Bhairi and Mohan (2007), anionic and cationic detergents have properties that enable them to disrupt protein-protein interactions. Glover *et al.* (1999) observed that detergents significantly increased cytoplasmic membrane fluidity of both Gram-positive *Staphylococcus aureus* and Gram-negative *Proteus mirabilis* in the order of non-ionic > cationic > anionic. Their investigation used probes to determine the effect of detergents on cell membranes and from their results determined that there was increased fluidity in both the outer and cytoplasmic membranes of *P. mirabilis* caused by interference of the tightly packed phospholipid hydrocarbons.

While it may be expected that an increase in cytoplasmic membrane fluidity would lead to cell death, no relationship was observed between the level of membrane fluidisation and biocidal activity and the biocidal efficiency of the detergents was observed to be dependent on the organism tested (Glover *et al.*, 1999). However, Chapman *et al.* (1993) stated that cationic surfactants alter the permeability of cell membranes leading to loss of function and cell death, depending on the extent of the effect.

Non-ionic detergents consist of a non-polar head group (Table 1.2) that is usually ethylene oxide units and a hydrocarbon chain and it is the proportion of hydrophilic and hydrophobic groups on the molecule that determine the properties (Moore and Payne, 2004). As a group the non-ionic surfactants

show little or no antimicrobial activity (Hugo, 1971) and are generally assumed to be inactive (Glover *et al.*, 1999; Moore and Payne, 2004). However, work by Brown and Richards (1964) observed that following treatment with the non-ionic detergent Polysorbate 80, *Pseudomonas aeruginosa* became more susceptible to the antibacterial activity of BAC. Work by Brown and Winsley (1969) then investigated whether the detergent caused alterations in cell permeability. They observed changes in membrane permeability caused by Polysorbate 80, which may have been due to the detergent disrupting the molecular structure of the cell envelope, which could lead to loss of cytoplasmic constituents or accumulation of substances toxic to the cell (Brown and Richards, 1964).

Middleton (2003) and Hayes (2006) also observed that after treatment with detergents, some cells became more susceptible to the action of disinfectant and Hugo *et al.* (2004) wrote that anionic and some non-ionic detergents may alter the permeability of the outer envelope to render some bacterial species more sensitive to antimicrobial agents. However, further research by Hayes (2006) revealed examples of a reduction in susceptibility of some cells to disinfectants, following treatment with detergent.

### 1.3.2 Disinfectants

Disinfectants are defined as agents that 'reduce micro organisms to an acceptable level which is harmful neither to health nor to the quality of perishable goods' (Fisher, 2003) and they can be bactericidal, sporicidal, fungicidal or a combination (Cerf *et al.*, 2010).

The safety of disinfectants is regulated by the European-wide scheme (The Biocidal Products Directive 98/8/EEC) which is implemented by the Biocidal Products Regulations (BPR). With the focus for safer foods and longer shelf-life (Langsrud and Sundheim, 1997), disinfectants are considered as essential in achieving the required hygiene status in food production areas (Meyer, 2006) and the regulations ensure that products available on the market do not cause harm to people, the environment or animals, and are effective (HPA, 2012). They are applied for a recommended contact time, after the application of detergent, to destroy remaining spoilage and pathogenic organisms that can arise from the environment, people and pests. Although it is normal for viable bacteria to be present following disinfectant application (Cerf *et al.*, 2010), failure to maintain a safe level of hygiene with the use of disinfectants can lead to food poisoning incidences, product recall and a loss of profit and reputation for the business concerned.

According to Holah, (1995), pre cleaning with detergents has been shown to reduce the number of bacteria on surfaces by 2-6 log orders however, considering bacterial numbers on surfaces have been observed by Holah *et al.* (1989) to be between  $10^7$  and  $10^{10}$  organisms  $\text{ml}^{-1}$ , and by Gram (2006) to be  $10^4$ - $10^6$  cfu  $\text{cm}^{-2}$ , viable bacteria are likely to remain on surfaces following

the cleaning procedure. Disinfection is therefore regarded as a crucial step in achieving the desired hygiene status in food production areas (Meyer, 2006) and the disinfectant chosen will be dependent on the nature of the industry, the types of microorganisms and the environmental conditions (Pasanen *et al.*, 1997; Bessems, 1998). The antimicrobial activity of a disinfectant is influenced by its chemical composition, presence of organic matter, in use concentration, temperature and pH (Lourenco *et al.*, 2009).

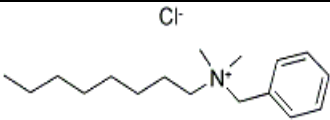
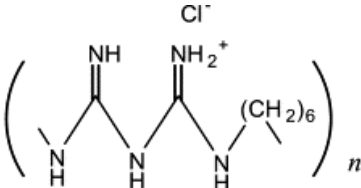
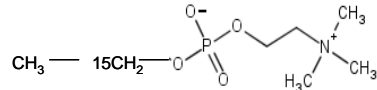
When investigating the efficacy of disinfectants, it is common to test under 'dirty' conditions by the addition of a protein load as it has been observed that the efficacy of some products is influenced or reduced in the presence of organic material (Cordier *et al.*, 1989). However, as the aim of this study was specifically to investigate the effect of detergents on the efficacy of disinfectants, all experiments were carried out without the addition of organic and / or inorganic materials. For food industry use the requirements of standard test protocols for contact time and temperature of disinfectants are 5 minutes and 20 °C (Bessems, 1998), which were followed in this study. Under these conditions the BS EN 1276:1997 suspension tests require that the disinfectants demonstrate at least a  $10^5$  reduction in viable counts.

Disinfectants can be divided into two main groups; oxidising disinfectants (Table 1.3) such as the chlorine releasing agents sodium hypochlorite, sodium dichloroisocyanurate (NaDCC) and hypochlorous, and non-oxidising disinfectants (Table 1.3.1) such as the quaternary ammonium compounds (QACs) which include benzalkonium chloride (BAC).

Table 1.3. Types and properties of oxidising biocides used in the food industry

Disinfectant	Active property	Mode of action	Structure	References
<b>Oxidising</b>				
Sodium hypochlorite	Hydrolyses to produce hypochlorous acid /hypochlorite ions depending on pH	Denature proteins and lipids leading to alterations in cellular metabolism. React with genetic material, oxidise organic material. Wide antimicrobial spectrum.	<p>Sodium dichloroisocyanurate</p>	Dukan <i>et al.</i> , (1999); Fisher(2003); Meyer (2006).
Iodophors	Iodine gradually released and free iodine acts as the disinfecting agent	Iodine penetrates the cell wall of microorganisms causing disruption of protein and nucleic acid structure and synthesis. Broad antimicrobial spectrum, less active against bacterial spores.	<p>• <i>xI</i></p>	Fisher(2003); Meyer (2006)
Peracetic acid	Combined with water produces acetic acid and hydrogen peroxide	Oxidizes and disrupts the outer cell membranes of microorganisms leading to cell death Wide antimicrobial spectrum.		Fisher (2003)
Hydrogen peroxide	Breaks down into water and hydrogen releasing free oxygen radicals	Oxidation of cell membranes. Bactericidal and fungicidal.	<p>H<sub>2</sub>O<sub>2</sub></p>	Fisher (2003)

Table 1.3.1 Types and properties of non-oxidising biocides used in the food industry

Non oxidising				
Quaternary ammonium compounds e.g. Benzalkonium chloride	Ionize in solution to produce a surface-active cation.	Interact with bacterial cell surfaces to disrupt and partially solubilize the cell wall. Destabilize the cytoplasmic membrane integrity leading to cell lysis.		Chapman, (2003); Gilbert and McBain (2003).
Biguanides	Cationic	Destabilize divalent cations of the cell envelope and disrupt the LPS and cause cell lysis. Binds to bacterial DNA and causes lethal damage. Equally active against Gram-positive and Gram Negative organisms.		Allen <i>et al.</i> , (2004); Chapman, (2003); Fisher (2003); Gilbert and McBain (2003).
Amphoterics	Ionize in solution to produce cations, anions or zwitterions depending on pH.	Active against Gram-negative and Gram-positive bacteria.		Fisher (2003); The Merck Index (2006)

Although all disinfectants are inactivated to some extent by organic soil, oxidising/chlorine releasing agents have the advantage of a broad spectrum of activity that includes application as a disinfectant in the food industry (Fisher, 2003), water treatment (Clasen and Edmondson, 2006) and human resistance to infection (Dukan *et al.*, 1999) and are unaffected by hard water.

QACs, which are predominantly used in farms, food manufacture, food transport and food retail sites (Holah *et al.*, 2002), have a narrower range and limited sporicidal activity.

Although the targets of antibiotics are quite specific, the mode of action of disinfectants involves multiple cellular targets (Poole, 2002; Maillard, 2002; Gilbert and McBain, 2003) including cell wall components, functional groups of proteins and genetic material (Meyer, 2006; Cerf *et al.*, 2010).

While previous investigations have shown that organisms in food processing environments are able to adapt to disinfectants through repeated exposure (Aase *et al.*, 2000; To *et al.*, 2002), few studies have yet investigated the possibility that pre exposure to detergent during normal cleaning procedures may affect the susceptibility of the organisms to the subsequent disinfectant treatment. If the susceptibility to a disinfectant is increased by detergent treatment it may mean that in use disinfectant concentration could be reduced. On the other hand, if susceptibility to a disinfectant is reduced, it could suggest an increase in contact time, an increase in concentration or a different combination of detergent and disinfectant are required.

#### **1.3.2.1 Oxidising agents**

Chlorine releasing agents include hypochlorites that oxidise organic material (Gardner and Peel, 2001; Chapman, 2003) and are common microbial agents in cleaning and sanitizing operations (Clasen and Edmondson, 2006) due to their high antimicrobial efficacy (Moore and Payne, 2004). They release free available chlorine (FAC) in the form of the hypochlorous acid (HOCl) (Clasen and Edmondson, 2006), which is a small, chemically reactive oxidising agent



that interacts indiscriminately with the bacterial cell (Maillard, 2002). Oxidising agents react with all organic molecules (Chapman, 2003) and destroy the molecular structure of cell proteins, which are an essential part of the structure of bacteria, viruses, yeast and fungi (Wainwright, 1988). They form substitution products with proteins and amino acids (Earnshaw and Lawrence, 1998) and are particularly active against the thiol groups of cysteine residues that are important in protein structure and function (Lambert, 2004). Destabilisation of the bonds between cysteine residues affects the folding and stability of the proteins in the cell wall and membrane (Narayan *et al.*, 2000). Both Gram-negative and Gram-positive bacteria are highly susceptible to the actions of chlorine releasing agents (Gardner and Peel, 2001) and they have a wide range of antibacterial activity against viruses (Moore and Payne, 2004) due to the multiple targets on the cell. Resistance to oxidising agents is achieved through inactivation of the agent or reduction in target access (Chapman, 2003).

NaDCC is a chlorine releasing disinfectant and activity is due to the release of HOCl (hypochlorite) by hydrolysis in aqueous solutions. It has a pH of between 7 and 9 at in-use concentration (Fisher, 2003; Clasen and Edmondson, 2006).

Bloomfield and Miles (1979) observed inactivation of greater than  $10^9$  organisms  $\text{ml}^{-1}$  of Gram-negative organisms *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klebsiella aerogenes* and Gram-positive *Staphylococcus aureus* with commercially available tablets containing 500 mg NaDCC that were dissolved to give solutions containing 125 ppm available chlorine. Their work demonstrated that activity of hypochlorite is determined

by a relationship between the total available chlorine that produces HOCl molecules and the concentrations of H<sup>+</sup> ions (pH). Their work also showed that NaDCC was significantly more active at pH 6 than at pH 9.6 which was agreed with by Moore and Payne (2004) who stated that hypochlorites are more active at acid pH which promotes hydrolysis of HOCl. Mazzola *et al.* (2003) recommended NaDCC for hospital applications due to its slow decomposition and liberation of HOCl, which make it an effective biocide against a wide range of bacteria.

Disinfectants are known to be inactivated by organic materials that interact strongly with hypochlorite and in industry cleaning this would result in a reduction in the bactericidal activity of chlorine containing compounds (Dychdala, 2001; Fisher, 2003).

#### **1.3.2.2 Quaternary ammonium compounds (QACs)**

QACs were first introduced in 1917 (Fisher, 2003) and are widely used as detergent-sanitizers in food environments (Gardner and Peel, 2001; Langsrud *et al.*, 2003). They are ammonium compounds with a monovalent cation (Table 1.3), are hydrophobic, have a high molecular weight (Mechin *et al.*, 1999) and for marked bacterial activity must have a chain length of between 8 and 18 carbon atoms (Fisher, 2003; Gorman and Scott, 2004). However, commercially produced BAC consists of homologs of different alkyl chain lengths mainly C12:C14 in a 60:40 ratio (Sutterlin *et al.*, 2008) as do commercially produced detergents such as the sodium alkyl sulphate product, SAS. QACs possess detergent properties and are cationic and

electrostatically attracted to the bacterial cell surface, which is hydrophilic and negatively charged (Frank and Koffi, 1990).

They are known as cationic surfactants that have strong bactericidal properties but weak detergent properties (Moore and Payne, 2004) as in solution they ionise to produce a cation, the substituted nitrogen part of the molecule, which provides the surface-active property (McDonnell and Russell, 1999; Fisher, 2003). However, QACs can be inactivated by the presence of anionic detergents and their antimicrobial activity reduced by non-ionic agents (Lehmann, 1988; Russell, 2004) and organic soil (Fisher, 2003). QACs are best used within their specific pH range and are more effective at alkaline and neutral pH than under acidic conditions (Moore and Payne, 2004).

Overall there has been much written about the target sites for QACs with the overall conclusion being that they cause general membrane damage to Gram-positive and Gram-negative bacteria (McDonnell and Russell, 1999; Russell, 2001). At alkaline pH, the number of negatively charged groups of the bacterial surface is increased giving the cationic QACs optimum interaction with the bacterial surface (Hugo, 1971; Gardner and Peel, 2001) to which they adsorb to and penetrate the cell wall causing damage and promoting their own uptake to disrupt the cytoplasmic membrane (Frank and Koffi, 1990; McDonnell and Russell, 1999). According to Skvarla *et al.* (2002) and Lukac *et al.* (2010) the cation is attracted to the negative charge of the cell membrane components where it interacts to cause general membrane damage. The hydrophobic tail of the molecule is then able to penetrate into the hydrophobic part of the cytoplasmic membrane. At low concentrations this leads to cytolytic leakage of cytoplasmic materials and at high

concentrations they cause coagulation of the cytoplasm (Kuda *et al.*, 2007). They are primarily active against Gram-positive bacteria with concentrations as low as 0.0005 % (v/v) being lethal (Moore and Payne, 2004) while higher concentrations are lethal to Gram-negative bacteria (Hamilton, 1971). Hugo (1971) wrote that QACs are more active at higher temperatures although Tuncan (1993) observed that efficiency of QACs above 100ppm did not vary with temperature. Their experiments dealt specifically with the effect of disinfectants on *Listeria*, due to its ability to grow in cold areas of food processing plants, and results showed that at low concentrations of 50 ppm, cold temperature (<7 °C) reduced the efficiency of QACs but there was no effect on efficiency at higher concentrations of 100–200 ppm. The antimicrobial action of disinfectants can be decreased by the presence of organic matter; Bessems (1998) reviewed the effect of practical conditions on the efficacy of disinfectants against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and noted that a high protein load did not affect the rate of kill of QACs or hypochlorite against the Gram-negative bacteria but significantly reduced the efficacy of the disinfectant against Gram-positive bacteria. However, this was dependent on the type of organism and the concentration of membrane active disinfectant against Gram-negative bacteria or oxidising disinfectant against Gram-positive bacteria. Overall however, QACs have a narrower spectrum than oxidising agents, with Gram-positive bacteria being more susceptible to the disinfectant than Gram-negative bacteria (Hugo, 1971; Gardner and Peel, 2001; Fisher, 2003).

### 1.3.2.3 Benzalkonium chloride

Benzalkonium chloride (BAC) is one of the most important and widely used quaternary ammonium compounds used for disinfection of surfaces in the food industry (Romanova *et al.*, 2007; Sutterlin *et al.*, 2008; Kuda *et al.*, 2011), and was used in this study. It causes alterations in membrane permeability that leads to leakage of cytoplasmic constituents from the cell (Kuda *et al.*, 2011).

### 1.3.2.4 Mode of action of disinfectants

The biocidal action of disinfectants occurs over four stages of interaction with the different components of the cell (Table 1.4) that ultimately leads to cell death.

Table 1.4. Interaction of disinfectants with cellular components.

Stages of interaction	Site of interaction	Effect of interaction	References
Uptake of disinfectant from solution	Adsorption to cell wall / outer membrane	Damage to outer cell layers, collapse of membrane potential, membrane disruption	Denyer and Maillard (2002); McDonnell and Russell (1999); Soumet <i>et al.</i> , (2005).
Penetration to target	Interaction with lipopolysaccharides and the phospholipids of the cytoplasmic membrane.	Damage to phospholipid bilayers and proteins. Membrane disorganisation and loss of integrity leading to leakage of low molecular weight material.	Denyer and Maillard (2002); McDonnell and Russell (1999).
Accumulation at target	Attacks cytoplasmic or inner membrane	Degradation of proteins and nucleic acids.	Denyer and Maillard (2002),
Interaction with target	Cytoplasm	Congeaing of cytoplasm	Denyer and Maillard (2002); McDonnell and Russell (1999).

The cell wall, cytoplasmic membrane and cytoplasm are all susceptible to disinfectant interaction (Denyer and Stewart, 1998) and to cause maximum damage, a disinfectant must cross the different outer layers of the Gram-positive or Gram-negative cell wall and penetrate the cell to reach its target. Most disinfectants are capable of acting on several sites within the cell with many disinfectants targeting bacterial membranes (McDonnell and Russell, 1999; Villalain *et al.*, 2001). Denyer and Stewart, (1998) state several modes of action of disinfectants that includes disruption of transmembrane proton motive force to inhibit metabolic reactions, loss of membrane integrity leading to leakage of intracellular contents and lysis and disruption of replication and coagulation of intracellular material. Russell and McDonnell (2000) agreed that due to the lack of specificity, and depending upon the concentration, disinfectants target several areas of the cell causing membrane damage, disruption of intermolecular interactions, disruption of tertiary structure and leakage of cytoplasmic components.

QACs are amphiphilic, cationic (positively charged) disinfectants that adsorb to the surface of the bacterial cell, damage the outer membrane of Gram negative bacteria and disrupt the cytoplasmic membrane which consequently promotes their own intracellular uptake and entry (Pasanen *et al.*, 1997; McDonnell and Russell, 1999; Gardner and Peel, 2001; Russell, 2002). This would presumably be by passive diffusion (McDonnell and Russell, 1999) following which damage would occur to the cytoplasmic or inner membrane. According to Earnshaw and Lawrence (1998), QACs react with the cell membrane to denature proteins and inactivate enzymes and Denyer and

Maillard (2002) reported that antibacterial agents target the cytoplasmic membrane or the cytoplasm to destabilize membranes, which leads to rapid cell lysis. The cytoplasmic membrane is the first target of antimicrobial agents entering the cell from the outside (Heinzel, 1988) and uptake of antimicrobial agents is the first stage of interaction (Denyer and Maillard, 2002). Interaction with the cell surface is determined by the physical characteristics of a disinfectant, such as charge or hydrophobicity, which determine its potential to penetrate the Gram-negative cell wall in order to interact with the target (Denyer and Maillard, 2002). This may be through hydrophobic interactions between the alkyl chain of the QACs and the fatty acid chains of membrane lipids, which causes disruption of the interactions between phospholipids, LPS and proteins (Denyer and Stewart, 1998; Lambert, 2004).

According to Russell and Gould (1988) it is the interaction of the hydrophobic part of the disinfectant with lipopolysaccharide and lipids that is key for entry to the cell and McDonnell and Russell (1999) agree that the primary target site appears to be the cytoplasmic (inner) membrane of bacteria where the long alkyl chain of the disinfectant disrupts the structural organisation and integrity of the cytoplasmic membrane.

While the molecular interaction between target and disinfectant may not be fully understood, the entire membrane can be considered as a target site (Chapman, 2003). Studies with protoplasts in suspension have shown that QACs induce lysis of protoplasts by causing generalised rather than specific membrane damage. It is also agreed by several authors that at low concentrations they affect membrane integrity and at high concentrations they cause congealing of the cytoplasm (Russell, 1986; McDonnell and Russell,

1999; Lambert, 2004). Cloete (2003) reported that high concentrations were required to effect antibacterial action and that the rate of penetration of the biocide to target site is dependent on concentration.

#### **1.4 Factors affecting susceptibility**

The bacterial cell wall of organisms acts as a permeability barrier to antibiotics and biocides (Russell, 2003a) and is the site where mechanisms of resistance to disinfectants are employed such as inactivation and reduction in target access and target alteration (Chapman, 2003).

##### **1.4.1 The cell wall and cytoplasmic membrane**

There are important differences in the cell wall structure in Gram-positive and Gram-negative organisms (Figure 1.3) and also within strains following response to adaptation to environmental conditions (Sikkema *et al.*, 1995). Both contain a rigid layer of peptidoglycan but while this is a relatively thick layer in Gram-positive bacteria, Gram-negative bacteria have a thin layer of peptidoglycan surrounded by an outer membrane of phospholipid and LPS. The cell wall is the site of many processes such as oxidative phosphorylation, active transport of solutes and ATP synthesis. ATP synthesis is driven by proton motive force (PMF) that is generated by the transfer of protons across the cytoplasmic membrane (Lambert, 2004) and by oxidation-reduction reactions occurring during electron transport. The cell wall surrounds the cytoplasmic membrane which is constructed of phospholipids, into which are inserted hydrophobic proteins. It is the site of many balanced interactions that



control permeability of the cell but also render it susceptible to attack by disinfectants (Denyer and Stewart, 1998).

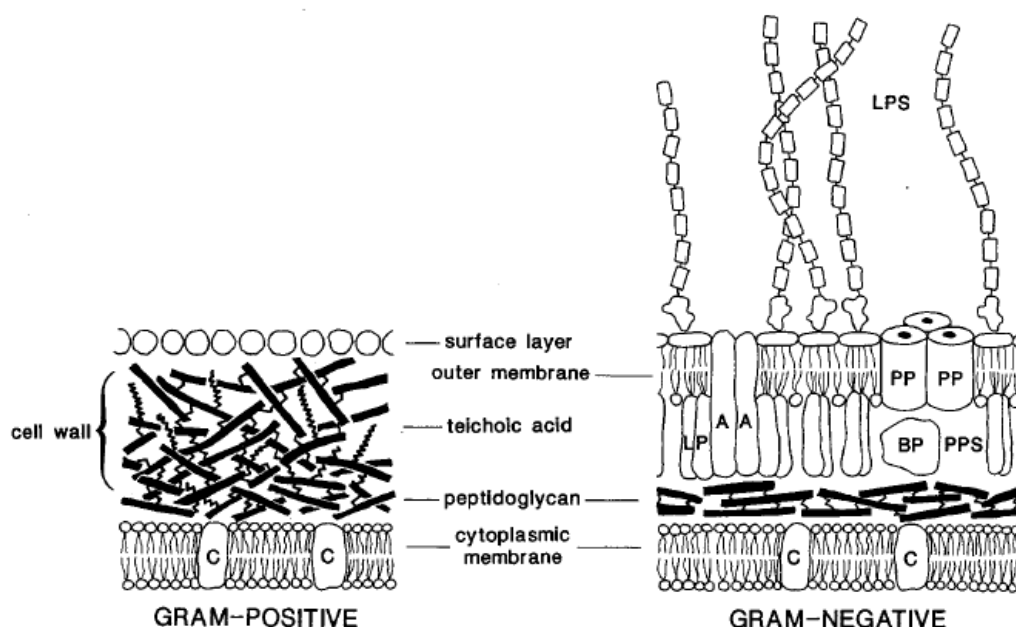


Figure 1.3. Structure of the cell envelope of Gram-positive and Gram-negative bacteria. PP, porin; C, cytoplasmic membrane embedded protein; BP, binding protein; PPS, periplasmic space; A, outer membrane protein; LP, lipoprotein (Sikkema *et al.*, 1995).

### 1.4.2 Hydrophobicity

The cell surface hydrophobicity of bacteria can vary between species and strains and can change according to growth conditions and composition of media (Briandet *et al.*, 1999; Li and McLandsborough, 1999). Cell surface hydrophobicity and electrochemical properties are important parameters in adhesion of bacteria to surfaces (Li and McLandsborough, 1999; Brown, 2005) and hydrophobic and electrostatic interactions are involved in maintaining the organisation of cell membrane components. Hydrophobicity is significant to the integrity of the cell membrane as alterations can affect permeability of the cell membrane, which can lead to cell death (Kim *et al.*,

2007). In a review by Sikkema *et al.* (1995), work was identified that shows the affinity for hydrophobic compounds is greater for bacteria with hydrophobic cell walls than those with hydrophilic cell walls suggesting that if interactions with a solute caused the cell surface to change from hydrophobic to hydrophilic, it would be protected against lipophilic compounds (van Loosdrecht, *et al.*, 1990; Jarlier and Nikaido, 1994).

Surfactants that interact with the cells may alter cell surface charge and alter the properties that define hydrophobic interactions (Brown, 2005) and these changes in cell surface hydrophobicity affect how bacteria respond to disinfectants (Maillard, 2007). Anionic surfactants adsorb to the negatively charged, lipophilic cell surface by their hydrophobic tails and the hydrophilic head groups orientate towards the aqueous environment, which increases the overall negative charge of the cell surface. Cationic surfactants adsorb via their positively charged head groups, which lowers the overall negative charge of the cell surface (Skvarla *et al.*, 2002). Park and So (2000) observed that an LPS mutant of *Bradyrhizobium japonicum* was more hydrophobic than the wild type strain which was attributed to absence of the O antigenic part of the LPS which agreed with observations made in an earlier study with *Serratia marcescens* (Bar-Ness *et al.*, 1988). When transformed with the LPS gene, wild type hydrophobicity was restored. They reported that changes in the LPS appear to effect surface properties such as cell surface hydrophobicity.

### 1.4.3 The Gram-negative outer membrane

Due to the unique character of the OM complex of the Gram-negative cell wall (Nixdorff *et al.*, 1978) that comprises lipoproteins and LPS, access of antimicrobial agents to the cell membrane is impeded (Gardner and Peel, 2001; Denyer and Maillard, 2002; Russell, 2004) as the OM acts as a permeability barrier and is responsible for intrinsic resistance to anti-microbial compounds (Maillard, 2002). Hamilton (1971) suggested that the Gram-negative cell envelope might constitute a non-absorbing barrier or, may absorb and retain, preventing passage to the inner cytoplasmic membrane. The LPS molecule, which is thought to play a role in maintaining OM integrity (Delcour, 2009), consists of three parts; the lipid, hydrophobic A region, which is anchored into the outer membrane (Al-Tahhan, *et al.*, 2000), a negatively charged core oligosaccharide region and the hydrophilic O antigen polysaccharide region (Lorinczy and Kocsis, 2001). The lipid A region contains a number of charged groups, most of them being anionic (Nikaido, 1996a), and the stability of the outer membrane is strongly dependent on cross linking by divalent cations including  $Mg^{2+}$  and  $Ca^{2+}$ , (Heinzel, 1988; Russell, 2003a). These react with negative charges on the phospholipid (Wainwright, 1998) and compensate for electrostatic repulsion between neighbouring LPS molecules (Delcour, 2009) to give integrity and strength of the outer membrane (Nikaido and Vaara, 1985). As the LPS is an amphiphilic molecule, it is able to bind both hydrophobic and hydrophilic compounds (Lorinczy and Kocsis, 2001). Also in the Gram-negative OM are many embedded proteins including porins, which are diffusion proteins (Delcour, 2009).

Russell (2003a) suggested that the most likely target sites for disinfectants would be the proteins in the outer membrane, which would sustain changes that effect membrane integrity. Although relatively permeable to small molecules, the outer membrane is not permeable to large or hydrophobic molecules (Allison and Gilbert, 2004) that are prevented from entering the membrane by the lipophilic LPS (Nikaido, and Vaara, 1985) and the strong interactions between LPS molecules and phospholipids (Nikaido, 1996a; Cloete, 2003) resulting in passage by diffusion across the outer membrane bilayer (McDonnell and Russell, 1999). However, the LPS provides a hydrophilic environment that is permeable to hydrophilic molecules of less than 600 g mol<sup>-1</sup>, that pass through water filled porins across the outer membrane (Nikaido, 1985; McDonnell and Russell, 1999; Al-Tahhan *et al.*, 2000; Denyer and Maillard, 2002) that impart the outer membrane with a low permeability to hydrophobic compounds.

Although Gram-negative organisms are more resistant to disinfectants such as QACs, due to their relatively impermeable outer membrane (Sundheim *et al.*, 1998; McDonnell and Russell 1999), alteration to the LPS can affect the susceptibility of Gram-negative bacteria to many types of antibiotics (Delcour, 2009) and biocides as the LPS is essential in maintaining the integrity and the membrane impermeability of the OM (Vaara, 1992; Denyer and Maillard, 2002). This was reported when Vaara and Vaara (1983) suggested that the positively charged antibiotic polymixin binds to the core and lipid A components of the LPS of *E. coli* or *S. typhimurium* to disorganise and increase permeability of the OM. This leads to strains being much more sensitive to a wide range of hydrophobic compounds including antibiotics and

detergents (Vaara, 1992) and suggests that this is a major component involved in maintaining the barrier property of the OM (Nikaido, 1996a).

#### **1.4.4 The Gram-positive cell wall**

Gram-positive bacteria are generally more sensitive to biocides than Gram-negative bacteria (Denyer and Maillard, 2002; Stickler, 2004) due to the composition of the cell envelope (Russell, 2004), which is the target for biocidal action, which can alter or destroy the essential cellular structure (Jordan *et al.*, 2008). The cell wall of Gram-positive bacteria consists of 90% peptidoglycan plus teichoic acids, polysaccharides and proteins (Russell, 2003a). Teichoic acids are important components of the cell wall (Jordan *et al.*, 2008) as they are mainly responsible for the overall negative net charge of the Gram-positive cell surface (Allison and Gilbert, 2004; Bhavsar *et al.*, 2004).

The cell wall regulates movement of essential nutrients across the membrane by specific transport systems and is the site of respiratory enzymes and coenzymes, which are important in cellular respiration, and in controlling metabolism in the cell (Singer and Nicholson, 1972; Heinzl, 1988), together with systems involved in cell wall synthesis (Gardner and Peel, 2001).

Russell (2003a) suggested that as large molecular weight polymers are able to enter the cell through the peptidoglycan and associated anionic polymers, it is doubtful that the cell wall would prevent the uptake of biocidal agents that need to cross the outer layers of the cell to reach the target site.

#### **1.4.5 The cytoplasmic membrane**

The cytoplasmic membrane is a phospholipid bilayer, with proteins inserted, and the composition of protein and lipid varies between cell types (Garcia – Saez and Schwille, 2010). The cytoplasmic membrane is protected by the OM in Gram-negative bacteria and the cell wall in Gram-positive bacteria and is the last barrier that separates the cytoplasm from the external environment (Kim *et al.*, 2007). It is selectively permeable and controls the influx of hydrophobic and high molecular weight compounds (Russell, 2003a; Garcia – Saez and Schwille, 2010). Target sites for disinfectants are often situated at the cytoplasmic membrane where disruption of can cause leakage of cell components including potassium, phosphates, nucleic acids and proteins (Lambert and Hammond, 1973), and can lead to physical disruption of the membrane, dissipation of proton motive force (PMF) and inhibition of membrane-associated enzyme activity (Maillard, 2002). Interactions such as ionic and hydrogen bonding and hydrophobic interactions help maintain stability and integrity of the membrane that is required in order to maintain diffusion across the membrane and to keep the phospholipid layer electrochemically balanced (Palsdottir and Hunte, 2004) however, this can be disrupted by membrane active agents that damage metabolic functions and cause leakage from the cytoplasm (Lambert, 2004).

#### **1.5 Resistance**

According to Cerf *et al.* (2010) the term resistance should be carefully defined. When considering disinfectant, resistance is the preferred term when killing is being studied, but tolerance is the preferred term when referring to adaptation

to inhibitory concentrations. In the food industry, resistance is the ability to survive short exposure to disinfectants (Sundheim *et al.*, 1998) and resistant microorganisms were described by Holah *et al.* (2002) as those that survived repeated cleaning and disinfection programmes to become the dominant flora. Chapman (1998) and Russell (2002) both reported that resistance to biocides was generally to concentrations below that used in industrial practice and Cloete (2003) says that resistant bacteria are those that are not susceptible to in use concentrations of antibacterial agents. According to Meyer (2006), resistance to disinfectants is regarded as low as long as disinfectants are used under appropriate conditions.

Heir *et al.* (1998) comment that the resistance of microorganisms to biocides can differ and it is difficult to define between tolerance and resistance. Meyer (2006) also states that only significant variations from the average should be regarded as resistance as susceptibility may be restored when the biocide is withdrawn (Russell, 2003b).

### **1.5.1 Intrinsic and Acquired resistance**

Bacterial resistance is of two types: intrinsic resistance, which is a chromosomally determined phenomenon (Cloete, 2003; Meyer, 2006), or acquired resistance, which is a phenotypic adaptation process that may occur through mutation or plasmid acquisition, and is not hereditary (McDonnell and Russell, 1999; Meyer, 2006).

#### **1.5.1.1 Intrinsic resistance**

Intrinsic resistance is regarded as a natural property of the cell (McDonnell and Russell, 1999) and a species is considered intrinsically resistant when it demonstrates a greater resistance than others (Meyer, 2006) that is common to all members of a given bacterial species (Sanchez *et al.*, 2009). It is dependent on the properties of the cells with the difference in structure of the cell walls of Gram-negative and Gram-positive bacteria imparting an inherent intrinsic resistance to help protect against unfavourable environmental conditions (McDonnell and Russell, 1999).

#### **1.5.1.2 Acquired resistance**

Acquired resistance causes phenotypic changes to occur in the cell causing certain strains to differ significantly in their susceptibility to biocides compared to others of the same species and can occur through mutation or plasmid acquisition. It can develop through genetic changes following exposure to sub lethal concentrations of disinfectants or short term exposure (Lunden *et al.*, 2003), as a result of incorrect concentrations being administered or, failure to remove organic matter that inactivates the disinfectant (Gelinas and Goulet, 1983), It may also arise through exposure of sensitive cells to antibacterial agents (Stickler, 2004) and can be avoided by strict cleaning regimes and use of disinfectants at sub lethal levels (Meyer, 2006).

McDonnell and Russell (1999) wrote that restricted entry of biocides into cells was due to membrane changes that were responsible for acquired resistance in Gram-negative bacteria which included changes in cell surface hydrophobicity, outer membrane ultra structure and outer membrane fatty acid



composition. Russell (1992) and Russell (2003b) suggested that resistance might be a result of reduced uptake of biocide through modification of target sites, activation of an efflux mechanism or inactivation of the biocide. Levin and Rozen (2006) state that phenotypical adaptation is not true resistance as it is not hereditary and is lost when organisms are not subjected to the biocide (Meyer, 2006). This was also reported by Jones *et al.* (1989) who observed that resistance to QACs was gradually lost in *P. aeruginosa* when the organisms were no longer grown in the biocide as did Mechin *et al.* (1999) who observed gradual loss of adaptive response of *P.aeruginosa* to a QAC following six subcultures in media without the disinfectant. Lunden *et al.* (2003) exposed persistent *L.monocytogenes* from a food-processing environment to increasing concentrations of QACs and sodium hypochlorite and observed increased MICs to both of the disinfectants. The increased resistance was due to adaptation to the disinfectants and decreased over a period of 28 days depending on the strain.

The resistance of organisms in biofilm demonstrates phenotypic adaptation (McDonnell and Russell, 1999) as growth is inhibited but cell death does not occur, and it is defined as a transient change in susceptibility (Chapman, 2003). It is not a change in the cells but the properties of the biofilm that confers resistance by inactivating the active property of disinfectants before they are able interact with the cells (Chapman, 2003). Cloete (2003) reported that possible mechanisms of resistance of bacteria in a biofilm as including interaction of the agent with the biofilm polymer and enzyme mediated resistance. In general, Gram-positive bacteria are sensitive to QACs (Sundheim *et al.*, 1998) but adherent *Listeria* cells are more resistant to

biocides than *Listeria* cells in suspension (Aarnisalo *et al.*, 2000; Frank and Koffi, 1990; Wirtanen and Mattilda-Sandolm, 1993) as the attachment of *L. monocytogenes* on surfaces impairs the efficacy of disinfectants (Aarnisalo *et al.*, 2007).

Sub lethal concentrations of disinfectants may also lead to attainment of selective genes through plasmid acquisition that may cause inactivation or decreased uptake of disinfectant, or code for efflux pumps (Soumet *et al.*, 2005), and the acquired resistance can be transferable (McDonnell and Russell, 1999; Russell, 2001). Mereghetti *et al.* (2000) observed an increase in resistance in *L.monocytogenes* strains to QACs but were unclear whether resistance was plasmid mediated. Earnshaw and Lawrence (1998) investigated the effect of disinfectants on the resistance of *L.monocytogenes* and did not observe any difference in resistance between strains that carried plasmids and those that did not, while in a review of bacterial resistance to disinfectants, Russell (1998) wrote of resistance mediated by plasmids in organisms such as *S. aureus*, *E. coli* and *S. marcescens*. Langsrud *et al.* (2004) stated that acquired resistance in *E. coli* and *Pseudomonas aeruginosa* was mainly related to changes in membrane composition while Aase *et al.* (2000) observed acquired resistance to BAC by *L. monocytogenes* through adaptation experiments that the resistance was due to an efflux pump of cationic surfactants. The mechanism of resistance in staphylococci and *L. monocytogenes* has been observed to be similar in both organisms with staphylococci containing plasmids that code for efflux membrane proteins and *Listeria* demonstrating a cross resistance with ethidium bromide (EtBr) that involved an active efflux mechanism (Soumet *et al.*, 2005).

### **1.5.2 Mechanisms of reduced sensitivity to disinfectants**

While the bacterial cell envelope itself forms a barrier against the actions of disinfectants, reduced susceptibility to disinfectants has been attributed to several mechanisms which may be through genetic alteration, by acquisition of new genetic information, or by phenotypic adaptation that confers resistance through inactivation of disinfectant, alteration to target site or reduction of access through efflux (McDonnell and Russell, 1999; Chapman, 2003). Changes in the cell envelope or a reduction in the size and number of porins act as barriers to penetration while modification of target sites means decreased accumulation of disinfectant (Maillard, 2007).

Cells adhered to surfaces may also be protected by food elements as Kuda *et al.* (2011) observed when egg yolk conferred resistance to *S. typhimurium* and *S. aureus* by protecting the cells from disinfectant treatment, and cells in biofilm are less sensitive (Russell, 1998; Gilbert *et al.*, 2001) as they are protected from the activity of disinfectants by biofilm components such as extracellular polysaccharides (Chapman, 2003).

While much of the literature relates resistance mechanisms to antibiotic resistance (Poole, 2002; Delcour, 2009; Martinez and Rojo, 2011), they may not apply to disinfectant resistance as bacteria have multiple target sites (Maillard, 2002) for disinfectant, and detoxifying enzymes are relatively unknown (Poole, 2002). This suggests that efflux maybe the main mechanism of reduced sensitivity to disinfectants and there has been much research to shown that resistance to QACs by staphylococci, *P. aeruginosa* and *L. monocytogenes* are through the presence of efflux pumps (Leelaporn

*et al.*, 1994; Heir *et al.*, 1999; Aase *et al.*, 2000; Poole, 2002). Gomez-Escalade *et al.* (2005) cited by Maillard (2007) also observed a decrease in susceptibility of *E. coli* to triclosan that they attributed to a combination of efflux and cell membrane impermeability suggesting that more than one mechanism may be involved.

#### **1.5.2.1 Efflux**

Some organisms are able to pump toxic molecules out of the cell by efflux pumps (Stickler, 2004) that are recognised as relevant mechanisms for resistance (Denyer and Maillard, 2002) as the phenotypic expression of an efflux pump reduces the susceptibility of organisms to biocides, which protects the cell and enhances resistance (Gilbert and McBain, 2003).

There are many literature references to efflux pumps in both Gram-positive (Lyon and Skurray, 1987; Heir *et al.*, 1998) and Gram-negative (Kazama *et al.*, 1998; Poole, 2005; Pos, 2009) organisms. Some antibiotic efflux systems, such as *E. coli* TetA, are specific to a single drug while others can transport a wide range of structurally and functionally unrelated compounds from the cell such as antibiotics, dyes and detergents. This protects the cell by limiting biocide uptake and accumulation and is leading to increasing concern of several organisms over their level of multi drug resistance (Poole, 2001).

The broad specificity can be a consequence of the hydrophobic nature of the transported molecules (Markham and Neyfakh, 2001). Both EtBr and BAC are removed by the same efflux pump in Gram-positive and Gram-negative bacteria (Heir *et al.*, 1998; Heir *et al.*, 1999; Aase *et al.*, 2000) that may be due to the structure of the molecules as both are monovalent cations (Aase *et*

*al.*, 2000).

Efflux pumps can be encoded by the chromosome or by plasmids (Piddock, 2006a; Romanova *et al.*, 2006) and are classified as multi drug resistance pumps (MDR) of which there are five families (Figure 1.4) based on amino acid sequence homology (Stavri *et al.*, 2007); the ATP binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic – compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family (Borges - Walmsley and Walmsley, 2001; Piddock, 2006a; Poole, 2005; Poole and Lomovskaya, 2006). Classification is based on whether the pump has single or multiple components, how many regions the transporter protein spans, the energy source for the pump and the type of substrate that the pump exports. Organisms are able to express MDR efflux pumps from more than one family or more than one pump belonging to the same family (Piddock, 2006b).

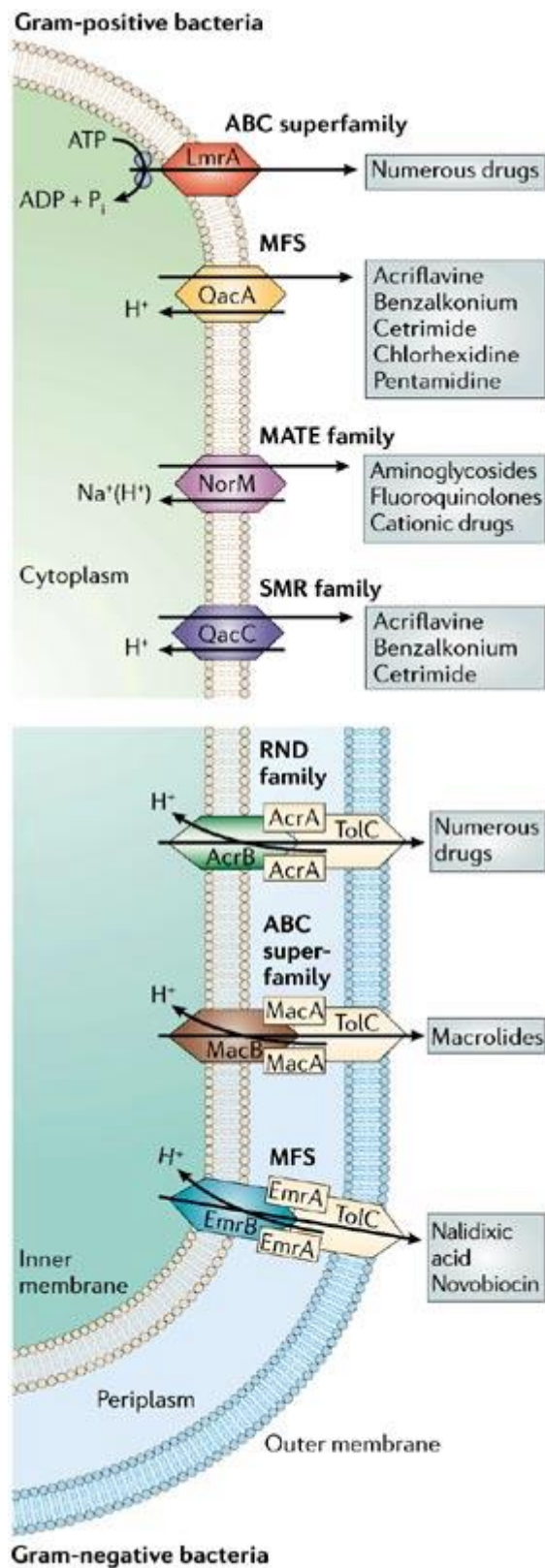


Figure 1.4. Multidrug resistance pumps in Gram-negative and Gram-positive bacteria (Piddock, 2006b).

The energy for efflux pumps in the MFS, SMR and RND families comes from proton driven antiporters that are generated by respiration. This produces an electrochemical gradient to transport the substrate where one H<sup>+</sup> ion is exchanged for one drug molecule (Paulson, 2003). The ABC superfamily hydrolyses ATP to drive efflux (Borges – Walmsley and Walmsley, 2001) while the MATE efflux pumps are driven by proton motive force (PMF) and the sodium ion gradient (Piddock, 2006b). MDR pumps are able to efflux a broad range of antimicrobial agents which has been explained as being due to a hydrophobic cavity present in the regulator protein that can accommodate different structures within the membrane or the periplasmic space, depending on the type of efflux pump present. The cavity has many hydrophobic residues that are able to bind with anionic or cationic substances via hydrogen bonding or electrostatic interactions to activate efflux (Paulson, 2003). The RND pumps have also been observed to have a hydrophobic region that binds substrates, with the Acr component being the major site for substrate recognition (Pos, 2009). Piddock (2006a) explains that there is controversy as to whether over expression of MDR efflux pumps gives rise to disinfectant resistance however, McMurray *et al.* (1998) observed that over expression of AcrB caused a two fold decreased susceptibility to triclosan by *E. coli*.

#### **1.5.2.2. Efflux in Gram-negative bacteria**

The low permeability of the Gram-negative OM is considered a barrier to hydrophobic agents however, entry of these agents to the cell can only be slowed down and additional mechanisms such as efflux are required for significant resistance (Li *et al.*, 1994; Nikaido, 1996b). Poole (2001) reported

that multidrug resistance in *P. aeruginosa* resulted from a synergy between OM impermeability and chromosomally encoded multidrug efflux pumps.

The efflux pumps expressed by *E. coli* and other Gram-negative bacteria are the most important factor in intrinsic and acquired resistance to antimicrobials (Poole and Lomovskaya, 2006) as they restrict build up of intracellular or periplasmic concentrations (Nikaido and Pages, 2012). Chromosomally encoded (Paulsen, 2001; Gilbert and McBain, 2003) or carried on plasmids they are organised in tripartite systems (Figure 1.5) that consists of a transporter protein in the inner cytoplasmic membrane (e.g. AcrB), a periplasmic protein (e.g. AcrA), with a central pore, that mediates between transporter and outer membrane protein, and an outer membrane protein (e.g. TolC) that forms a channel in the outer membrane for exit of substrates and is driven by proton motive force. The AcrB transporter belongs to the RND family and transports substrates such as short chain fatty acids, SDS and Triton X from the inner membrane of the bacterial cell envelope or the cytoplasm, to the external medium via TolC (Eswaran *et al.*, 2004) while another known *E. coli* efflux system, EmrAB, belongs to the MFS family and has been observed to show resistance to hydrophobic toxins such as carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (Tanabe *et al.*, 2009).



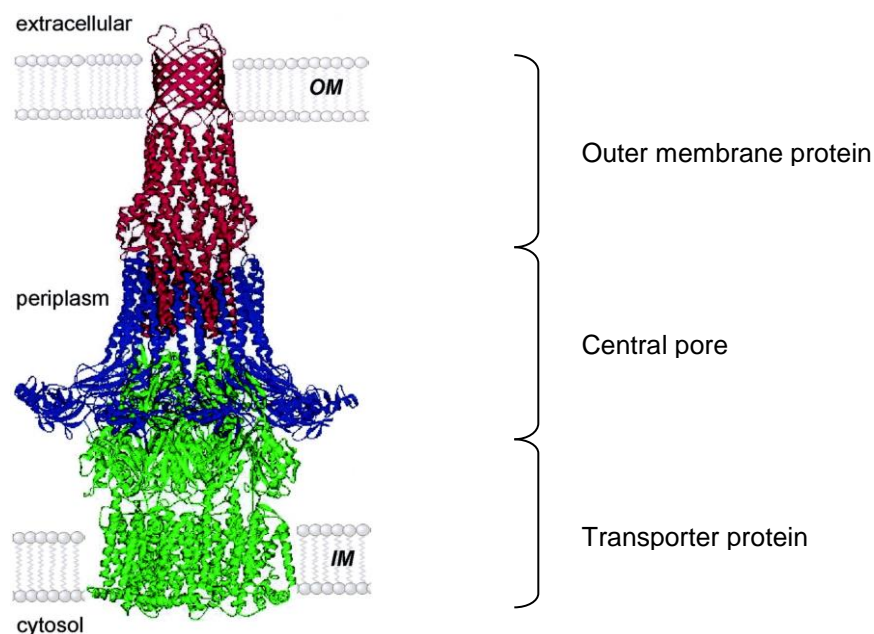


Figure 1.5. Possible model of tripartite efflux pump. Spanning the outer membrane is TolC and the inner membrane is AcrB. The figure shows MexA (a close homologue of AcrB) spanning the periplasm. (Piddock, 2006a)

Some classes of antibiotics have no useful activity against Gram-negative bacteria because of the presence of RND pumps in these organisms (Piddock, 2006a; Poole and Lomovskaya, 2006).

### 1.5.2.3. Efflux in Gram-positive bacteria

Gram-positive bacteria express single cytoplasmic membrane transporters (Stavri *et al.*, 2007) that efflux various drugs and several efflux mechanisms (Figure 1.4) have been described (Markhan and Neyfakh, 2001; Paulsen *et al.*, 2001; Poole, 2002). The ABC super family utilises energy from ATP hydrolysis to efflux drugs out of the cell against the concentration gradient while MFS and SMR transporters exchange drug molecules for protons using the transmembrane electrochemical gradient (Markham and Neyfakh, 2001).

Staphylococci utilise MDR transporters that are driven by PMF (Sundheim *et al.*, 1998; Poole, 2002). The ability of Gram-positive organisms to efflux a broad range of toxins may be due to the hydrophobic nature of the molecules (Markham and Neyfakh, 2001) that bind to and activate the regulator proteins of the multidrug transporters that efflux hydrophobic cations from the cell (Ahmed *et al.*, 1994). However, although an MdrL pump that extrudes antibiotics and EtBr and an Lde pump that is associated with fluoroquinolone resistance have both been described in *L. monocytogenes*, they are not considered to be sufficient to confer disinfectant resistance to the organism (Romanova *et al.*, 2006).

#### **1.5.2.4. Efflux pump inhibitors Iza and Glynn**

The observed increase in resistance to antimicrobials by organisms such as *Pseudomonas aeruginosa* and *E. coli*, particularly those with clinical relevance, has led to many investigations into the potential of EPIs to restore susceptibility (Pages *et al.*, 2005; Stavri *et al.*, 2007; Babajide *et al.*, 2011). EPIs can work in several ways to inhibit the efflux of compounds, which may be through blocking the channel in the outer membrane, disruption of energy source or alterations to the pump assembly. Several classes of efflux pump inhibitors and their mechanisms have been described that inhibit particular families of efflux pumps such as RND and MFS, or types of efflux pumps such as tetracycline Tet(B) and quinolone resistance in *P.aeruginosa* (Pages *et al.*, 2005).

One of the first inhibitors of the RND efflux pumps to be described was phenylalanine-arginine- $\beta$ -naphthylamide (PA $\beta$ N) that is thought to compete

with fluoroquinolones for efflux pumps (Bohnert *et al.* 2010), increase the activity of levofloxacin in *P.aeruginosa* and OM permeability at high concentrations (Pages *et al.*, 2005). (PAβN) was also observed by Cortez – Cordova and Kumar (2011) to inhibit the RND pump AdeFGH of *Acinetobacter baumannii*.

Other EPIs have been described including 1 – (1 – naphtylmethyl) – piperazine (NMP) that blocks RND pumps by competitive inhibition (Lomovskaya *et al*, 2001, Pannek *et al*, 2006). The EPI carbonyl cyanide *m* – chlorophenylhydrazone (CCCP) uncouples oxidative phosphorylation (Pages *et al.*,2005) and dissipates PMF by dissolving the membrane that become more permeable to H<sup>+</sup> ions (Aase, 2000), while *N,N* – dicyclohexyl – carbodiimide (DCCD) is an inhibitor of the F<sub>0</sub>F<sub>1</sub> ATPase (Lambert, and Le Pecq,1984).

In this study the EPIs reserpine and CPZ were used to determine whether efflux was a mechanism of decreased susceptibility in *L.monocytogenes* because previous studies have demonstrated their relevance to efflux systems in other Gram-positive organisms. Reserpine is a plant alkaloid that has been widely used in studies of antimicrobial agents and has been observed to be active against the MDR transporter NorA of *S. aureus* (Shhmitz, 1998; Stavri, *et al.*, 2007), the Tet(K) of methicillin resistant *Staphylococcus aureus* and the Bmr tetracycline efflux pump of *Bacillus subtilis*, by interacting with particular amino acid residues of a reserpine binding site. Chlorpromazine (CPZ) inhibits the binding of calcium to proteins that are essential in ATPase activity and energy production for efflux (Martins

*et al.*,2011) and has been observed to significantly increase the susceptibility of *Mycobacterium avium* to erythromycin (Rodrigues *et al.*, 2008)

## **1.6 Aim and Objectives**

The aims of this project are to investigate the effects of detergents such as SAS and FAE on susceptibility of *E. coli* and *L. monocytogenes* to disinfectants and to establish combinations that influence susceptibility to disinfectant. When combinations have been established, this investigation aims to determine the mechanisms that cause changes in susceptibility to occur.

## **CHAPTER 2            Materials and Methods**

### **2.1. Cultures**

Food industry isolates *Escherichia coli* CRA400 and *Listeria monocytogenes* CRA359 were provided by Campden and Chorleywood Food Research Association (CCFRA, Gloucestershire, UK). These organisms were chosen for this research as previous work by Hayes (2006) observed changes in their susceptibility to disinfectant following treatment with commercially used detergents.

### **2.2. Maintenance of organisms**

The organisms were transferred from frozen stock to 50 µl of Tryptone Soya Broth (TSB) (Lab M, UK) to rehydrate overnight then spread onto Tryptone Soya Agar plates (TSA) at 37 g l<sup>-1</sup> (Lab M, UK) and maintained at 4 °C. They were sub cultured onto Tryptone Soy Agar plates and incubated overnight at 37 °C when single colonies were required. Cell cultures were grown in 500 ml conical flasks containing 200 ml of sterile Tryptone Soya Broth (TSB) at 30 g l<sup>-1</sup>. The TSB was inoculated with a single colony of the required organism and was incubated for 24 hours, in an orbital shaker (Series 25, New Brunswick Scientific, UK), at 37 °C and 150 revolutions per minute (rpm).

### **2.3. Assessment of susceptibility**

#### **2.3.1. Stainless steel coupons**

Stainless steel coupons type 316 that measured 8 mm x 8 mm x 1 mm were supplied by CCFRA. To prepare the coupons for attachment of organisms

they were washed in Fairy (UK) liquid to remove oil and dirt from the cutting process. The coupons were rinsed in water then autoclaved at 121 °C for 15 minutes.

### **2.3.2. Preparation of cells for attachment to stainless steel surfaces**

An aliquot of 50 ml of overnight culture was centrifuged at 2000 x g for 30 minutes then the pellet was resuspended in 50 ml of Ringers solution. The resuspended cells were centrifuged again at 2000 x g for 30 minutes then the pellet was resuspended in 50 % (25 ml) of original volume of attachment media (TSB). The steel coupons were then immersed in the suspension and incubated at 20 °C for 1 hour for the cells to attach to the surface. After 1 hour (+ / - 10 seconds), the coupons were removed from the media and gently washed in Ringers solution to remove unattached cells.

### **2.3.3. Ringers solution**

Ringers tablets (Lab M, UK) were diluted in distilled water (1 tablet / 500 ml) as required then sterilised at 121 °C for 15 minutes.

### **2.3.4. Detergents**

Holchem Laboratories Ltd (Lancashire, UK) provided the anionic detergents sodium alkyl sulphate (SAS) and sodium lauryl ether sulphate (SLES) and, the non-ionic detergents fatty alcohol ethoxylate (FAE) and polyethoxylated alcohol (PEA). Sodium dodecyl sulphate (SDS) was obtained from Sigma – Aldrich. The detergents were prepared to the recommended working concentrations of 0.2 % (v/v), 0.2 % (v/v), 0.1 % (v/v), 0.2 % (v/v) and 0.2 %

(w/v) respectively, by dilution with sterile distilled water, immediately prior to use.

#### **2.3.4.1 Mass Spectrometry**

Detergents and carbon chain length standards were analysed by mass spectrometry (MS) to determine carbon chain lengths of the hydrocarbon tails. All MS analyses were performed on a Thermo Scientific Direct Probe Contoller – Firmware Version 2.1 ISQ Quadruple Mass Spectrometer (Thermo Scientific, UK) using Xcalibur 2.1.0.1140 software. The mass range was set at 50 – 1000 a.m.u with a dwell time of 0.1 seconds and ion source temperature was 250 °C.

#### **2.3.4.2 Formaldehyde**

As surfactants are mostly vegetable derived and prone to spoilage, formaldehyde is added to SAS as a preservative at a concentration of 0.07 % (v/v). The organisms were exposed to Formaldehyde (Sigma-Aldrich, UK), at this concentration, to ensure it did not have an effect on viability and membrane permeability. Results not shown.

#### **2.3.5. Disinfectants**

Benzalkonium chloride (BAC) provided by Holchem (Lancashire, UK) was used at a concentration of 0.01 % - 0.03 % (v/v) for *E. coli* and 0.005 % (v/v) for *L. monocytogenes*. This was lower than the recommended in use concentration but gave a measurable level of kill at which changes in susceptibility could be quantified. Sodium Dichloroisocyanurate (NaDCC) (Sigma – Aldrich, UK) and was used at a concentration of 0.0005 % for *E. coli* and 0.0008 % (w/v) for *L. monocytogenes*. The disinfectants were diluted in

water of standard hardness (WOSH), which was made in accordance with the British Standard Institute suspension test, BS EN 1276:1997 (Anon 1997).

### **2.3.6. Water of standard hardness**

WOSH was prepared in accordance with the British Standard Institute suspension test, BS EN 1276:1997 (Anon 1997). To make WOSH 19.84 g of anhydrous magnesium chloride (Sigma – Aldrich, UK) and 46.24 g of anhydrous calcium chloride (Lancaster Synthesis Ltd, UK) were dissolved in 1 l of distilled water to make solution A, which was autoclaved at 121 °C for 15 minutes. Solution B was made by dissolving 35.02 g of sodium hydrogen carbonate (Prolabo, UK) in 1 l of distilled water, which was sterilised by passing through a filter pore size 0.22 µm. 6 ml of solution A and 8 ml of solution B was added to a volumetric flask containing 600 ml of sterile distilled water (SDW) which was made up to a final volume of 1 l with SDW. The pH was adjusted to 7.0 + 0.2 and solutions A and B were stored at 4 °C for up to one month.

### **2.3.7. Effects of detergents on susceptibility of attached cells to disinfectant**

The stainless steel coupons with cells attached were immersed into detergent solution for 20 minutes at room temperature (approximately 20 °C). The coupons were then transferred to a petri dish, containing approximately 20 ml Ringers solution, then washed by gently swirling in the Ringers solution to remove excess or unbound detergent. The coupons were then immersed in disinfectant for 5 minutes and washed again in Ringers solution in a new petri dish. Control coupons were treated with SDW, detergent only or disinfectant only and the exposure times were selected to simulate industry practice. To



quantify the effect of the treatments, the surfaces were swabbed with sterile cotton swabs that were vortexed in 5 ml Ringers solution for 30 seconds. This was serially diluted in Ringers solution and plated on TSA plates that were incubated overnight at 37 °C.

### **2.3.8. Preparation of cells for suspension tests**

An aliquot of 40 ml of overnight culture was centrifuged at 5000 x g for 15 minutes. The pellet was then resuspended in 20 ml Ringers solution and the suspension was centrifuged again at 5000 x g for 15 minutes. The final pellet was resuspended in 25 ml sterile WOSH (in 100 ml flask). The cells were then ready to be used for disinfectant susceptibility testing, membrane permeability testing or hydrophobic interaction chromatography (HIC).

#### **2.3.8.1. Suspension tests to determine effect of detergent on susceptibility of cells to disinfectant**

The required amount of detergent to achieve working concentration (172 µl SAS or SDS, 185 µl SLES, 25 µl FAE) was added to the flask, which was incubated for 20 minutes, at room temperature, on a Luckham Rotostat shaker at 100 rpm. The detergent was then removed from the cells by centrifugation at 5000 x g for 15 minutes. The pellet was resuspended in 20 ml Ringers and centrifuged again at 5000 x g for 15 minutes. The final pellet was resuspended in 25 ml Ringers solution. Control flasks had water added instead of detergent and underwent the same procedures. To determine whether the detergent had an effect on the susceptibility of the cells to disinfectant, BAC was added to a concentration, that would give a measurable level of kill, of 0.05 % for *E. coli* and 0.0008 % for *L. monocytogenes* and the flasks were incubated at 20 °C, for 5 minutes, on a Luckham Rotostat shaker

(Luckham Ltd, UK) at 100 rpm. An aliquot of 1 ml of the suspension was then added to 8 ml of neutraliser (30 g l<sup>-1</sup> Tween 80S (v/v) (Sigma – Aldrich, UK), 3 g l<sup>-1</sup> lecithin (BDH Laboratory supplies, UK), 5 g l<sup>-1</sup> sodium thiosulphate (BDH Laboratory supplies, UK), 1 g l<sup>-1</sup> L– histidine (Sigma – Aldrich, UK), 30 g l<sup>-1</sup> saponin (BDH Laboratory supplies, UK) made in 1 l of phosphate buffer at 0.0025 mol l<sup>-1</sup>) and 1 ml of SDW. A 0.25 mol l<sup>-1</sup> phosphate buffer solution was prepared by dissolving 34 g of potassium dihydrogen orthophosphate (BDH Laboratory supplies, UK) in 500 ml distilled water. This was adjusted to pH 7.2 +/- 0.2 with 1 M NaOH and made up to 1 l with distilled water before sterilization by autoclave. The sterilized stock solution was diluted to 1 % (v/v) in SDW for preparation of the neutralizer. The neutralizer was not used for attached cells as the detergent was removed by rinsing. Two controls were required to ensure that the neutraliser did not affect cell viability or inactivate the disinfectant. For control A, 8 ml of neutraliser, 1 ml of SDW and 1 ml of bacterial suspension was added to a test tube and vortexed. After 5 minutes the test tubes were vortexed again and samples of the suspension were serially diluted and plated on TSA plates that were incubated overnight at 37 °C. For control B, 9 ml of disinfectant and 1 ml SDW were added to a test tube and vortexed. After 5 minutes, 1 ml was removed and added to a test tube containing 8 ml of neutraliser, which was then vortexed. After 5 minutes 1 ml of bacterial suspension was added to the test tube which was then vortexed again. After 5 minutes samples of the suspension were serially diluted and plated on TSA plates that were incubated overnight at 37 °C.

## **2.4 Investigation into mechanisms of detergent induced changes in disinfectant susceptibility**

### **2.4.1. Effects of detergents on cell membrane permeability**

Cells were prepared as previously for suspension tests. When the detergent had been rinsed from the cells, membrane integrity was determined by uptake of the fluorochrome propidium iodide (PI, Sigma-Aldrich, UK). Propidium iodide (PI) is a dye for nucleic acids, which is unable to pass through intact membranes due to its double positive charge, but is able to pass through membranes that have become permeable. Following passage through the membrane it interchelates to double stranded nucleic acids which increases its fluorescence by 20 to 40 times (Papadimitriou *et al.*, 2006; Shapiro, 2008). An aliquot of 10  $\mu$ l of PI (1 mg ml<sup>-1</sup> in water) was added to 1 ml of suspended cells that were analysed on a BD FACS Calibur Flow Cytometer (B. D. Biosciences, UK) which measured forward scatter (FS), side scatter (SS) and fluorescence (FL3) at 635 nm which is emitted by PI – stained cells (Ananta *et al.*, 2004). The same method was also used to determine effects on membrane integrity using a Perkin Elmer LS-5 Luminescence Spectrometer (Perkin Elmer, UK) with excitation and emission wavelengths of 520 and 590 nm respectively.

### **2.4.2. Hydrophobic interaction chromatography**

Cell surface hydrophobicity (CSH) was determined by hydrophobic interaction chromatography (HIC) using the methods of Smyth *et al.* (1978) with Sepharose CL-4B (Sigma-Aldrich, UK) as the non hydrophobic control and Octyl Sepharose (Sigma-Aldrich, UK) with hydrophobic ligand. Columns were prepared from glass Pasteur pipettes (Scientific Laboratory Supplies, UK) that

were plugged with a small amount of fibreglass. Silicone tubing (Sterilin, Ltd, UK) was attached to the pipette and this was fitted with a screw clip to control flow. Sepharose or Octyl Sepharose gel beads (0.6 ml) were added to the columns and were washed with 10 ml of 1 M ammonium sulphate to remove preservatives from the gel beads (Smyth *et al.*, 1978). The cells were prepared, as for suspension tests. After centrifugation and washing to remove the detergent, 100  $\mu$ l of suspension was added to 4.9 ml of 1 M ammonium sulphate and the absorbance was read at 540 nm on a CECIL1000 series bench spectrophotometer (Cecil Instruments, UK). An aliquot of 100  $\mu$ l of bacterial suspension was then slowly added to the prepared columns giving the cells time adsorb to the gel beads. The gel beads were then washed with 5 ml 1M ammonium sulphate and the absorbance of the eluate was taken for later comparison of adsorption and desorption. The cells were desorbed from the columns by the addition of 10 ml of 10 mM sodium phosphate buffer (pH 6.8) (Smyth *et al.*, 1978), which decreased the ionic strength. The flow of the buffer through the column was controlled by the gateway clamp and was maintained at 1–2 ml min<sup>-1</sup>. The eluate was serially diluted and plated out on TSA plates that were incubated overnight at 37 °C. The absorbance of the eluate was also taken to compare to previous readings. Changes in hydrophobicity were determined by calculating the log<sub>10</sub> difference in total viable count (TVC) of untreated and treated cells eluted from the sepharose and octyl sepharose columns.

### 2.4.3. Efflux

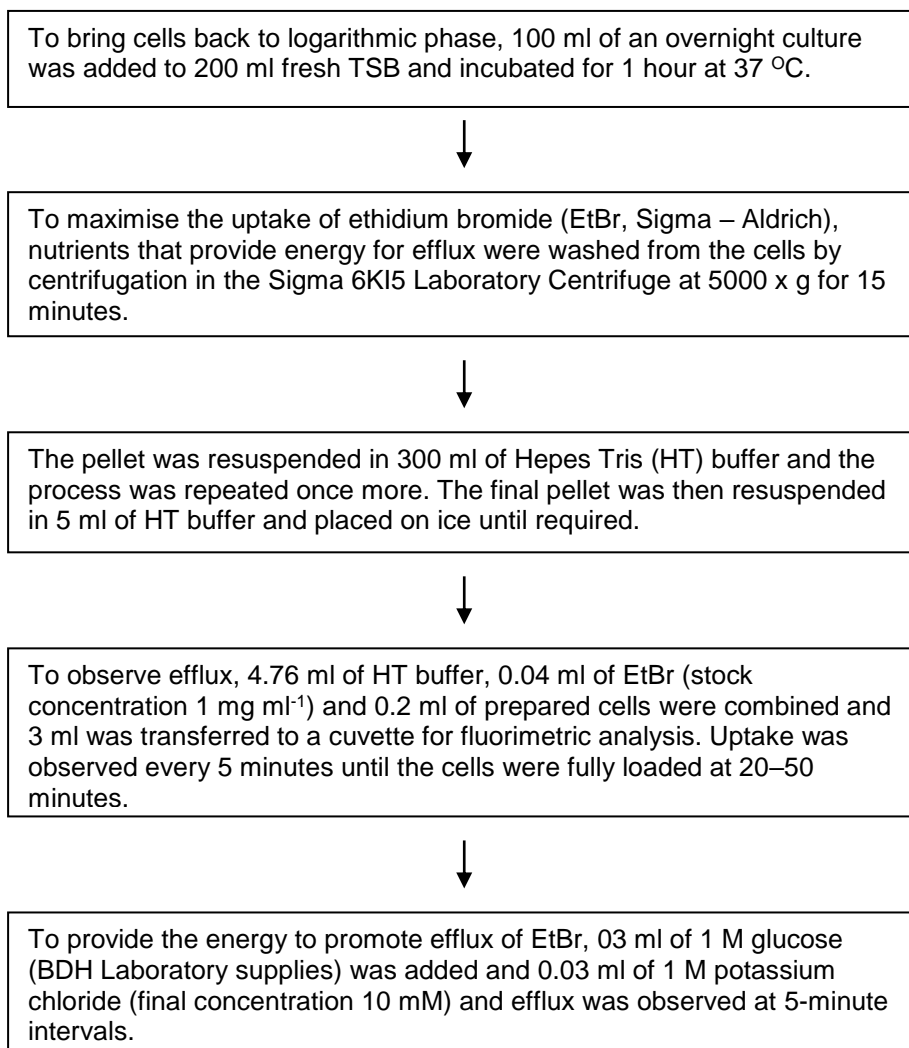


Figure 2.1. Process of preparation of *L. monocytogenes* for uptake and efflux of EtBr from the cells.

For further studies, detergent or the efflux pump inhibitors chlorpromazine (CPZ) and reserpine (both from Sigma-Aldrich) were also added, at the same time as glucose, to working concentrations.

#### 2.4.3.1. Efflux pump inhibitors

Efflux pump inhibitors (EPIs) were provided by Sigma-Aldrich and were made freshly as required to concentrations determined by MIC experiments. A 50

times concentrated stock solution of CPZ was made by dissolving 1.25 g of CPZ in 1 l of water which was then added to samples to a working concentration of 25 ml l<sup>-1</sup>. A 50 times stock solution of Reserpine was prepared by dissolving 31 mg of Reserpine in 0.5 ml of acetic acid which was then made up to 31.25 ml with water and added to samples to a working concentration of 20 mg l<sup>-1</sup>.

#### **2.4.4. Effects of carbon chain length on susceptibility of *E. coli* to BAC**

To determine whether the different carbon chain lengths in the detergents were having an effect on susceptibility or permeability of *E. coli*, cells were exposed to individual carbon standards (C10 sodium n-decyl sulphate, C12 sodium dodecyl sulphate, C14 sodium 1- tetradecyl sulphate, C16 sodium n-hexadecyl sulphate, C18 sodium n-octadecyl sulphate) (Alfa Aesar, UK). Cells were prepared as for attachment and suspension tests and were exposed to the carbon standards for 20 minutes, which is the same time as exposure to the commercial detergents. Effects on susceptibility were determined by TVC and for permeability by flow cytometric analysis using PI.

#### **2.5. Statistics**

Results were analysed using Minitab 15, t-test or ANOVA with significance determined by a 95 % confidence level with a p-value of < 0.05 being significant.

### 3.1 Investigations in to the effect of detergents on the susceptibility of *E. coli* and *L. monocytogenes* to disinfectants

Experiments were carried out to determine the effects of different detergents on the susceptibility of *E. coli* and *L. monocytogenes* to BAC. The organisms were allowed to attach to stainless steel for 1 hour after which they were exposed to either the detergents or water control for 20 minutes, rinsed in Ringers solution to remove unattached cells and detergent, and treated with either the disinfectant for 5 minutes or water as a control. The surfaces were swabbed and TVC determined for treated and untreated cells.

#### 3.1.1 Effect of detergent treatment on susceptibility to BAC

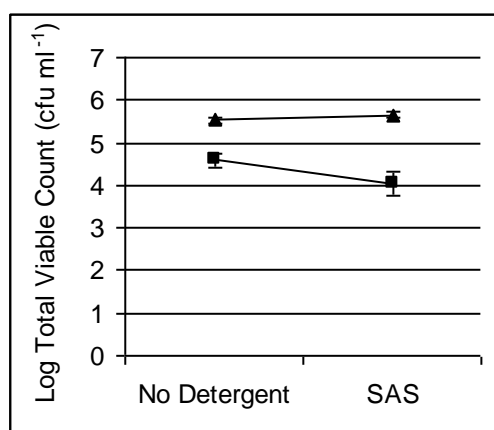


Figure 3.1. Interaction plot of *E. coli* treated with SAS followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=54. SE shown.

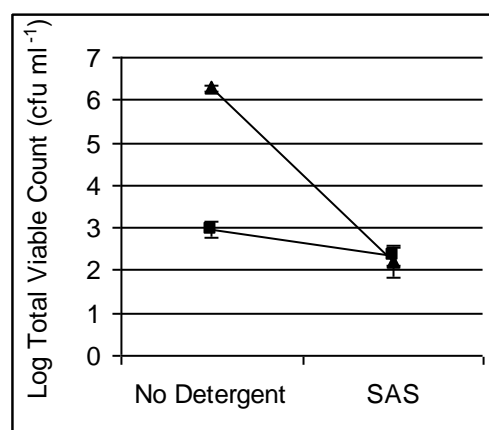


Figure 3.2. Interaction plot of *L. monocytogenes* treated with SAS followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.1 and 3.2 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent SAS for 20 minutes followed by BAC for 5 minutes. For *E. coli* (Figure 3.1) it can be seen that the TVC for untreated cells was Log 5.52

and treatment with SAS alone had no significant effect as the TVC was Log 5.65 ( $p=0.15$ ) while treatment with BAC alone resulted in a significant reduction ( $p<0.01$ ) in TVC to Log 4.56 (by 0.96 Log). Combined treatment of detergent followed by disinfectant significantly reduced ( $p=0.02$ ) TVC to Log 4.05 (by 1.47 Log) and the significant interaction effect between detergent and disinfectant results in a plot divergence that shows *E. coli* becomes significantly more susceptible to BAC following treatment with SAS. The plot divergence is very significant as it shows that the combined effect of detergent and disinfectant is value added and the overall increase in susceptibility is greater than the sum of the individual effects.

For *L. monocytogenes* (Figure 3.2) the TVC for untreated cells was Log 6.28 which was significantly reduced to Log 2.35 (by 3.93 Log) following treatment with SAS alone ( $p<0.01$ ) and to Log 2.94 (by 3.34 Log) by BAC alone ( $p<0.01$ ). The combination of detergent and disinfectant significantly reduced ( $p<0.01$ ) TVC to Log 2.22 (by 4.06 Log) and the effect of the interaction gives a plot convergence that shows *L. monocytogenes* becomes significantly less sensitive to BAC following treatment with SAS.

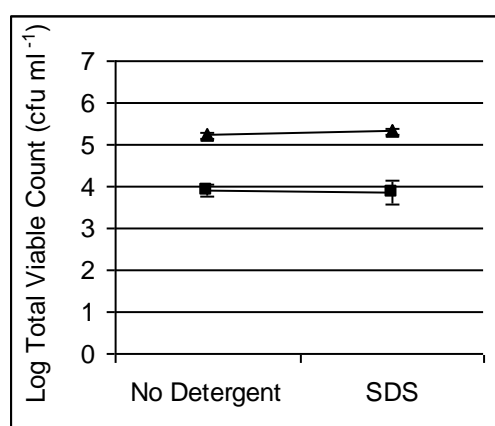


Figure 3.3. Interaction plot of *E. coli* treated with SDS followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=27$ . SE shown.

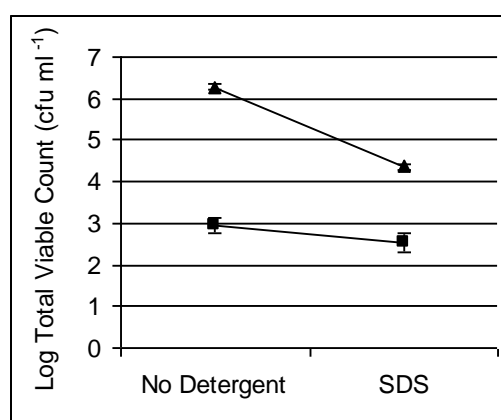


Figure 3.4. Interaction plot of *L. monocytogenes* treated with SDS followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=24$ . SE shown.



Figures 3.3 and 3.4 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent SDS for 20 minutes followed by BAC for 5 minutes. For *E. coli* (Figure 3.3) it can be seen that the TVC for untreated cells was Log 5.22. Treatment with SDS alone had no effect as the TVC was Log 5.32 ( $p=0.9$ ) while treatment with BAC alone significantly reduced ( $p=0.04$ ) TVC to Log 3.9 (by 1.32 Log). Treatment with detergent followed by disinfectant reduced TVC to Log 3.85 (by 1.37 Log) and showed no significant interaction effect of detergent combined with disinfectant ( $p=0.72$ ).

For *L. monocytogenes* (Figure 3.4) the TVC for untreated cells was Log 6.28 which was significantly reduced ( $p<0.01$ ) to Log 4.36 (by 1.92 Log) following treatment with SDS alone and to Log 2.96 (by 3.32 Log) with BAC alone. The combination of detergent and disinfectant significantly reduced ( $p<0.01$ ) TVC to Log 2.53 (by 3.75 Log) and the effect of the interaction gives a plot convergence that shows *L. monocytogenes* becomes significantly less sensitive to BAC following treatment with SDS.

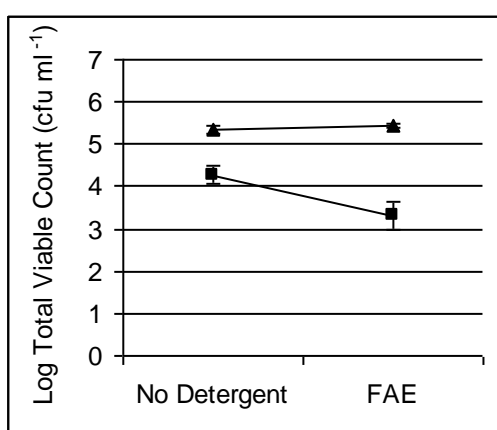


Figure 3.5. Interaction plot of *E. coli* treated with FAE followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=36$ . SE shown.

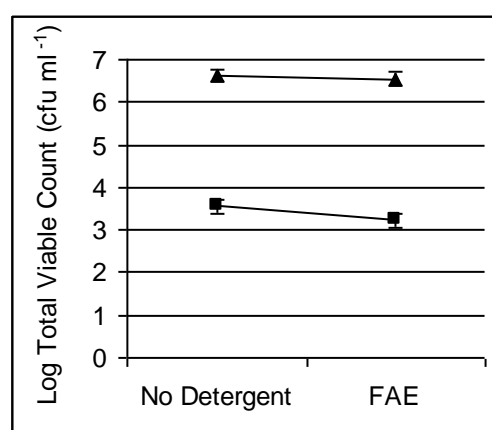


Figure 3.6. Interaction plot of *L. monocytogenes* treated with FAE followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=18$ . SE shown.

Figures 3.5 and 3.6 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent FAE for 20 minutes followed by BAC for 5 minutes. For *E. coli* (Figure 3.5) it can be seen that the TVC for untreated cells was Log 5.34. There was a small increase in TVC following treatment with FAE alone to Log 5.44, which may have been due to replication of cells during the 20 minutes, while treatment with BAC alone significantly reduced ( $p=0.04$ ) TVC to Log 4.26 (by 1.08 Log). Treatment with detergent followed by disinfectant significantly reduced ( $p=0.02$ ) TVC to Log 3.3 (by 2.04 Log) and the effect of the interaction gives a plot divergence that shows *E. coli* becomes significantly more susceptible to BAC following treatment with FAE.

For *L. monocytogenes* (Figure 3.6) the TVC for untreated cells was Log 6.63 and there was no significant effect of treatment with FAE alone ( $p=0.35$ ). BAC alone reduced TVC to Log 3.55 (by 3.08 Log) while the combination of detergent and disinfectant reduced TVC to Log 3.22 (by 3.41 Log). The results show that there was no interaction between the detergent and disinfectant that had any effect on TVC ( $p=0.52$ ).

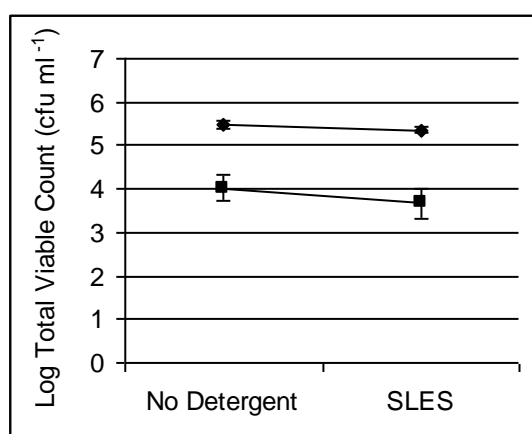


Figure 3.7. Interaction plot of *E. coli* treated with SLES followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=27$ . SE shown.

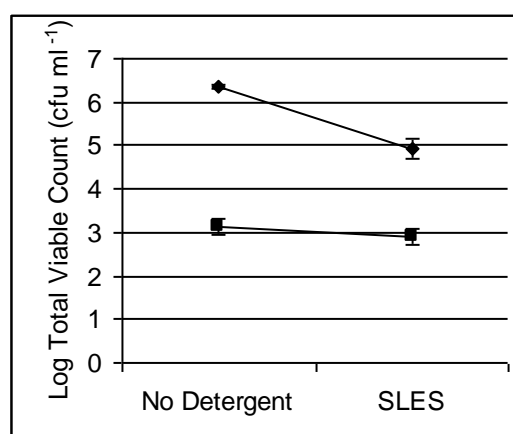


Figure 3.8. Interaction plot of *L. monocytogenes* treated with SLES followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=27$ . SE shown.

Figures 3.7 and 3.8 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent SLES for 20 minutes followed by BAC for 5 minutes. For *E.coli* (Figure 3.7) it can be seen that there was no significant effect on TVC following treatment with SLES alone ( $p=0.24$ ). Treatment with BAC alone significantly reduced ( $p<0.01$ ) TVC to Log 4.02 (by 1.47 Log) while treatment with detergent followed by disinfectant reduced TVC to Log 3.66 (by 1.83 Log) and showed no significant interaction effect of the combination ( $p=0.57$ ).

For *L. monocytogenes* (Figure 3.8) it can be seen that the TVC for untreated cells was Log 6.35 and there was a significant reduction ( $p<0.01$ ) to Log 4.91 (by 1.44 Log) following treatment with SLES alone and to Log 3.12 (by 3.23 Log) with BAC alone ( $p<0.01$ ). Treatment with detergent followed by disinfectant significantly reduced ( $p<0.01$ ) TVC to Log 2.9 (by 3.45 Log) and the effect of the interaction gives a plot convergence that shows *L. monocytogenes* becomes significantly less susceptible to BAC following treatment with SLES.

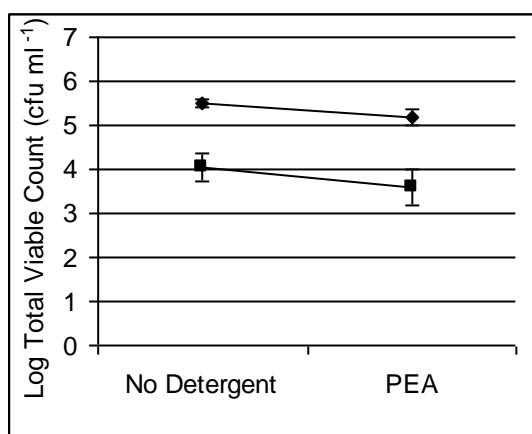


Figure 3.9. Interaction plot of *E. coli* treated with PEA followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=21$ . SE shown.

Figure 3.9 shows the effects of treating *E. coli* with the detergent PEA for 20 minutes followed by BAC for 5 minutes. It can be seen that there was no significant effect on TVC following treatment with PEA alone ( $p=0.12$ ) and a significant reduction to Log 4.02 (by 1.47 Log) following treatment with BAC alone ( $p<0.01$ ). Detergent followed by disinfectant reduced TVC to Log 3.58 (by 1.82 Log) but there was no interaction effect observed by the combination ( $p=0.76$ ).

### 3.1.2 Effect of detergent treatment on susceptibility to NaDCC

Experiments were also carried out to determine the effects of different commercially used detergents on the susceptibility of *E. coli* and *L. monocytogenes* to NaDCC which is an oxidising disinfectant that is also used in the food industry.

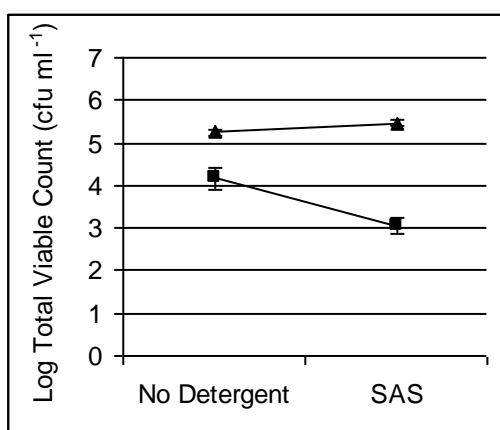


Figure 3.10. Interaction plot of *E. coli* treated with SAS followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=54$ . SE shown.

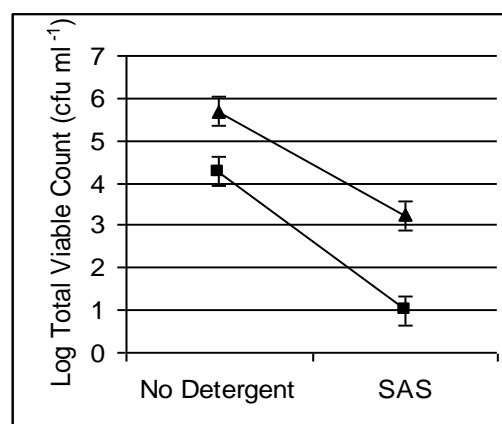


Figure 3.11. Interaction plot of *L. monocytogenes* treated with SAS followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=18$ . SE shown.

Figures 3.10 and 3.11 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent SAS for 20 minutes followed by NaDCC for 5 minutes.

For *E. coli* (Figure 3.10.) it can be seen that the TVC for untreated cells was Log 5.24. Treatment with SAS alone had no effect as the TVC was Log 5.47 while treatment with NaDCC alone significantly reduced ( $p < 0.01$ ) TVC to Log 4.17 (by 1.07 Log). Treatment with detergent followed by disinfectant significantly reduced ( $p < 0.01$ ) TVC to Log 3.07 (by 2.17 Log) and the effect of the interaction gives a plot divergence that shows that *E. coli* becomes significantly more susceptible to NaDCC following treatment with SAS.

For *L. monocytogenes* (Figure 3.11) the TVC for untreated cells was Log 5.68 and this was significantly reduced to Log 3.23 (by 2.45 Log) following treatment with SAS. NaDCC significantly reduced TVC to Log 4.26 (by 1.42 Log) while the combination of detergent and disinfectant significantly reduced TVC to Log 1.0 (by 4.6 Log). The results show an interaction effect of the detergent and disinfectant in combination and a divergence of the lines show that *L. monocytogenes* becomes significantly ( $p < 0.01$ ) more susceptible to NaDCC following treatment with SAS.

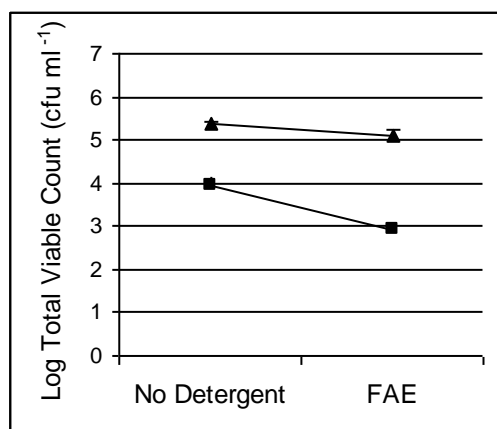


Figure 3.12. Interaction plot of *E. coli* treated with FAE followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=54$ . SE shown.

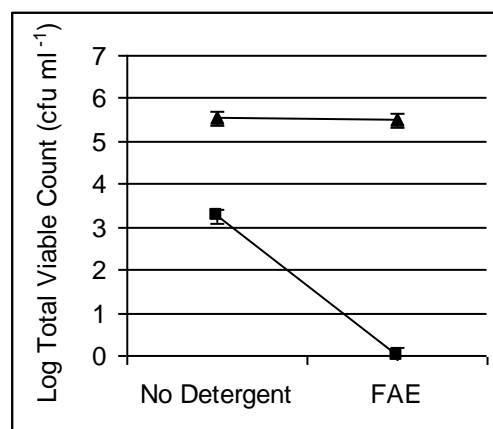


Figure 3.13. Interaction plot of *L. monocytogenes* treated with FAE followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=27$ . SE shown.

Figures 3.12 and 3.13 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent FAE for 20 minutes followed by NaDCC for 5 minutes. For *E. coli* (Figure 3.12) it can be seen that the TVC for untreated cells was Log 5.37 and there was no significant on TVC of treatment with FAE alone while treatment with NaDCC alone significantly reduced TVC ( $p < 0.01$ ) to Log 3.95 (by 1.42 Log). Treatment with detergent followed by disinfectant significantly reduced ( $p = 0.02$ ) TVC to Log 2.94 (by 2.43 Log) and the effect of the interaction gives a plot divergence that shows *E. coli* becomes significantly more susceptible to NaDCC following treatment with FAE.

For *L. monocytogenes* (Figure 3.13) the TVC for untreated cells was Log 5.54. FAE had no effect on TVC while treatment with NaDCC alone reduced TVC to Log 3.25 (by 2.29 Log). The combination of detergent and disinfectant significantly reduced ( $p < 0.01$ ) TVC to 0 (by 5.54 Log) and the effect of the interaction gives a plot divergence that shows *L. monocytogenes* becomes significantly more susceptible to NaDCC following treatment with FAE.

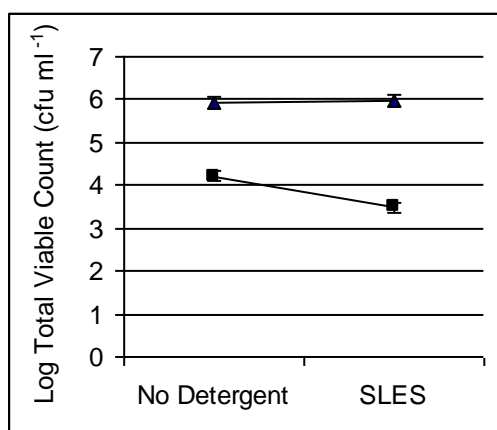


Figure 3.14. Interaction plot of *E. coli* treated with SLES followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=27$ . SE shown.

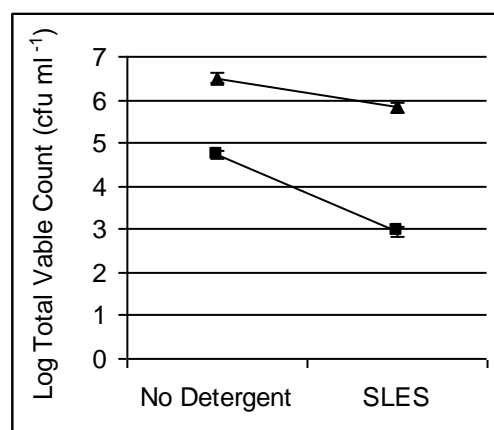


Figure 3.15. Interaction plot of *L. monocytogenes* treated with SLES followed by NaDCC. ▲ = no disinfectant, ■ = disinfectant.  $n=21$ . SE shown.

Figures 3.14 and 3.15 show the effects of treating *E. coli* and *L. monocytogenes* with the detergent SLES for 20 minutes followed by NaDCC for 5 minutes. For *E. coli* (Figure 3.14) it can be seen that the TVC for untreated cells was Log 5.92 and treatment with SLES alone had no effect on TVC while treatment with NaDCC alone significantly reduced TVC ( $p<0.01$ ) to Log 4.22 (by 1.7 Log). Treatment with detergent followed by disinfectant significantly reduced ( $p<0.01$ ) TVC to Log 3.48 (by 2.44 Log) and the effect of the interaction gives a plot divergence that shows *E. coli* becomes significantly more susceptible to NaDCC following treatment with SLES.

For *L. monocytogenes* (Figure 3.15) it can be seen that the TVC for untreated cells was Log 6.51 and there was no significant effect on TVC of treatment with SLES alone while treatment with NaDCC alone significantly reduced ( $p<0.01$ ) TVC to 4.72 Log (by 1.79 Log). Detergent followed by disinfectant reduced TVC to Log 2.95 (by 3.56 Log) demonstrating an interaction effect between the detergent and disinfectant with plot divergence showing that *L. monocytogenes* becomes significantly more susceptible ( $p<0.01$ ) to NaDCC following treatment with SLES.

### **3.1.3 Summary of results**

When comparing all of the results obtained so far (Table 3.1), it can be seen that no trend is observed for cells treated with detergents followed by BAC; while *E. coli* demonstrates an increase in susceptibility following treatment with anionic SAS and non-ionic FAE, SDS, SLES and PEA have no effect on susceptibility of the organism to BAC. *L. monocytogenes* demonstrates a

reduction in susceptibility following treatment with all of the anionic detergents but FAE does not have any effect.

When *E. coli* and *L. monocytogenes* are treated with detergents followed by NaDCC, a decrease in susceptibility to NaDCC is observed following treatment with anionic SAS and SDS and non-ionic FAE.

Table 3.1. Summary of the effect on susceptibility to disinfectant of *E. coli* and *L. monocytogenes* following treatment with detergent. + = observed increase in susceptibility to disinfectant, - = observed decrease in susceptibility to disinfectant, O = no effect on susceptibility observed, blank cells = not done.  $p < 0.05$  for all.

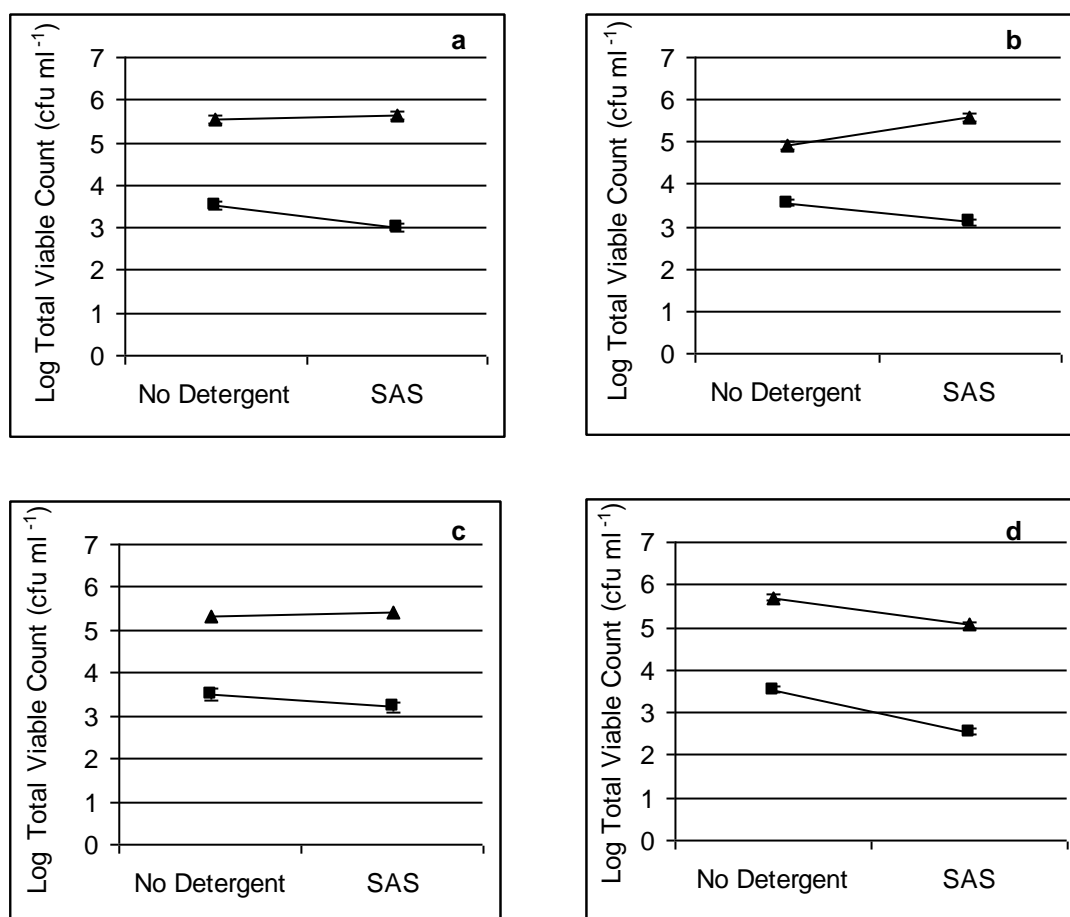
	Anionic detergents			Non-ionic detergents	
<b>BAC</b>	<b>SAS</b>	<b>SDS</b>	<b>SLES</b>	<b>FAE</b>	<b>PEA</b>
<i>E. coli</i>	+	O	O	+	O
<i>L. monocytogenes</i>	-	-	-	O	
<b>NaDCC</b>					
<i>E. coli</i>	+		+	+	
<i>L. monocytogenes</i>	+		+	+	

## 3.2 Time course experiments

### 3.2.1. Time course analysis of susceptibility of *E.coli* treated with SAS up to 4 hours followed by BAC.

It was previously observed that *E. coli* becomes significantly more susceptible to BAC following 20 minutes treatment with SAS (Figure 3.1) and FAE (Figure 3.5). As these detergents are used in food industry cleaning procedures, experiments were conducted to further explore the effects of the detergents by increasing the time of exposure.





Figures 3.16 a–d. Interaction plots of *E. coli* treated with SAS for; a = 20 minutes, b = 1 hour, c = 2 hours and d = 4 hours, followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.16 a-d show that treating *E.coli* with SAS alone for 20 minutes, 1 hour and 2 hours had no effect on TVC ( $p < 0.05$ , 0.14 and 0.023 respectively). but TVC significantly decreased following treatment with SAS alone at 4 hours ( $p < 0.01$ ). BAC alone causes a significant reduction in TVC ( $p < 0.01$  for all times). A significant interaction effect ( $p < 0.01$  for both) showing increased susceptibility to BAC was observed at 20 minutes (Figure 3.16a) and 1 hour (Figure 3.16b) but no interaction ( $p = 0.1$  for both) was observed at 2 hours or 4 hours (Figures 3.16 c and d respectively).

### 3.2.2 Comparison of effect of SAS and SDS for 2 hours on susceptibility of *E. coli* to BAC

In the previous experiments, *E. coli* were treated with detergent for 20 minutes before the disinfectant treatment and it can be seen that although the anionic detergent SAS caused a reduction in susceptibility of *E. coli* to BAC (Figures 3.1), anionic SDS had no effect (Figure 3.3). To investigate this further, *E. coli* cells were treated with the detergents for 2 hours, followed by treatment with BAC, to determine whether increased time of exposure to the detergents would have any further effects on susceptibility.

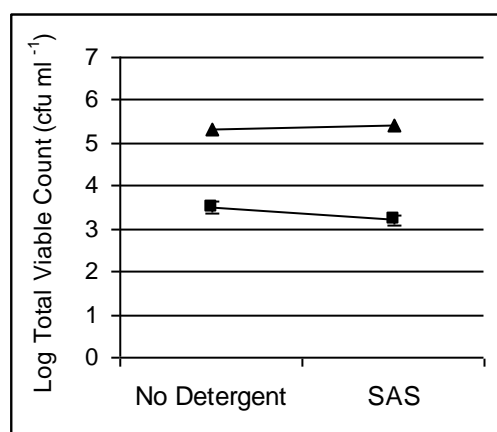


Figure 3.17. Interaction plot of *E. coli* treated with SAS for 2 hours followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=18.

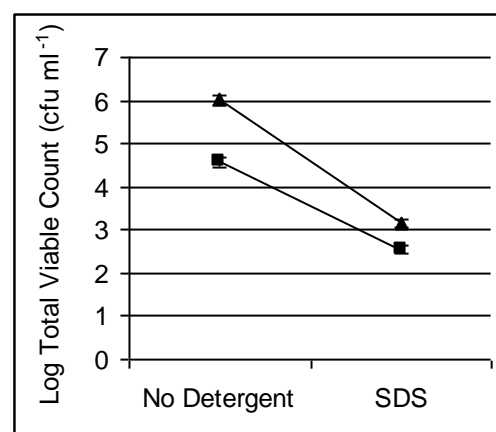


Figure 3.18. Interaction plot of *E. coli* treated with SDS for 2 hours followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=18. SE shown.

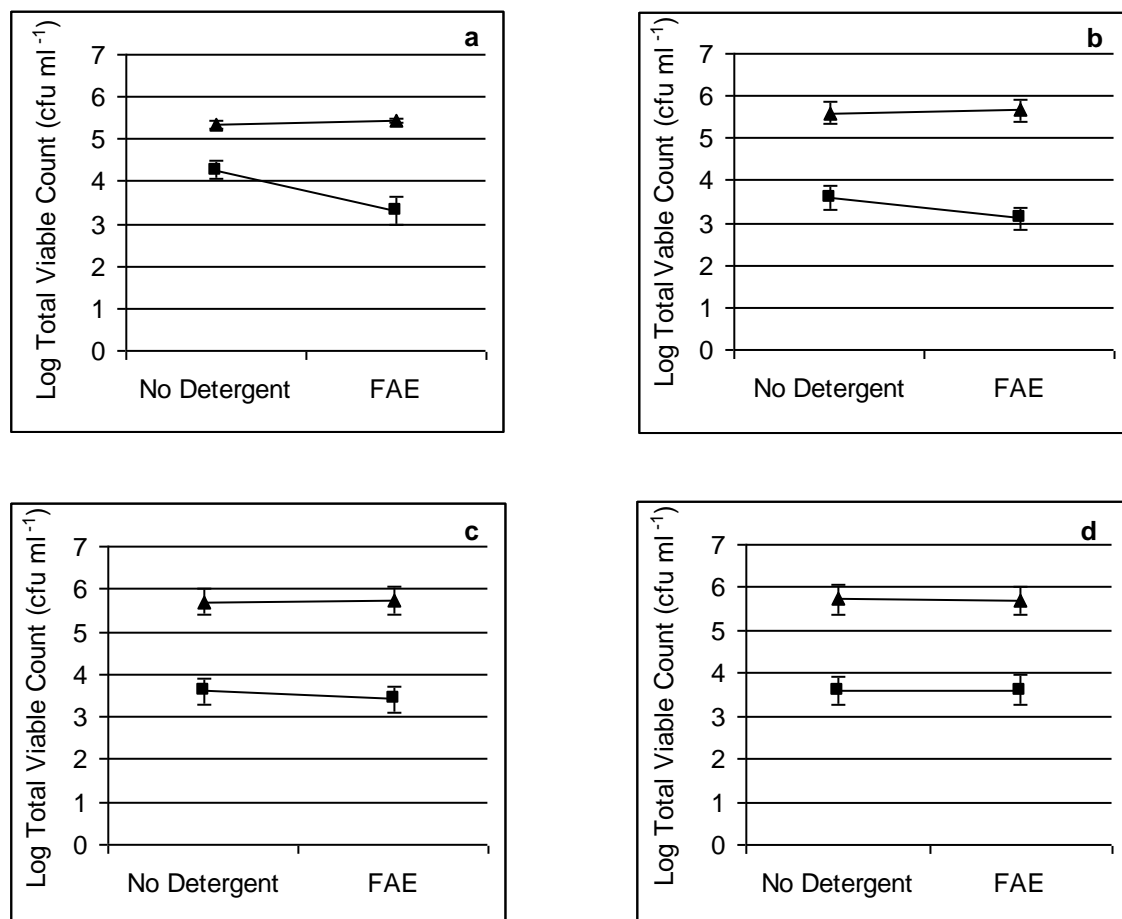
Figures 3.17 and 3.18 compare the effects of treating *E. coli* with the anionic detergents SAS and SDS for 2 hours followed by BAC. In Figure 3.17 it can be seen that SAS has no effect on TVC of *E. coli* ( $p=0.06$ ) while BAC reduced TVC significantly ( $p<0.01$ ). Following treatment with detergent and disinfectant, no significant interaction effect was observed ( $p=0.3$ ).

In Figure 3.18 it can be seen that both detergent and disinfectant treatments alone significantly reduced TVC ( $p<0.01$  for both). Following treatment with

combined detergent and disinfectant, an interaction effect was observed and a plot convergence shows that *E. coli* became significantly ( $p < 0.01$ ) more resistant to BAC following 2 hours treatment with SDS.

### 3.2.3 Time course analysis of susceptibility of *E. coli* treated with FAE up to 4 hours followed by BAC.

The experiments were continued to assess the effect of increased time of exposure to FAE on susceptibility of *E. coli* to BAC.



Figures 3.19 a-d. Interaction plots of *E. coli* treated with FAE for; a = 20 minutes, b = 1 hour, c = 2 hours and d = 4 hours, followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.19 a-d show that over 4 hours the results consistently show that detergent has no effect on TVC ( $p=0.5$  for all) while BAC alone causes a

significant reduction in TVC ( $p < 0.01$  for all). Following treatment with detergent and disinfectant in combination, figure 3.19a shows that *E. coli* becomes significantly more susceptible to BAC following 20 minutes ( $p = 0.4$ ) treatment with FAE but there is no significant interaction effect ( $p = 0.2, 0.05$  and  $0.8$  respectively) of the detergent and disinfectant at all other times (Figures 3.19 b-c).

### 3.2.4 Time course analysis of susceptibility of *L. monocytogenes* treated with SAS up to 2 hours followed by BAC.

Although *L. monocytogenes* exhibits a reduction in susceptibility to BAC following treatment with SAS (Figure 3.2), experiments were carried out to determine whether susceptibility would be further reduced if the cells were treated with the detergent for a longer period of 2 hours.

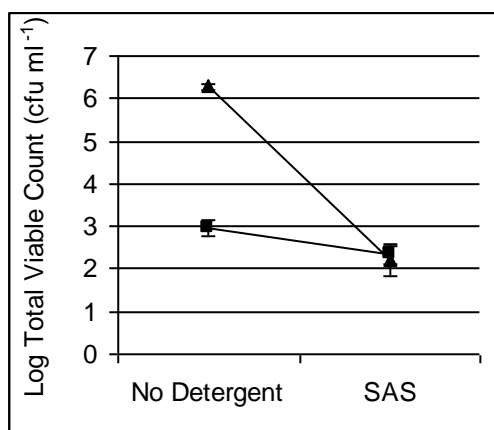


Figure 3.20. Interaction plot of *L. monocytogenes* treated with SAS for 20 minutes followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=9$ . SE shown.

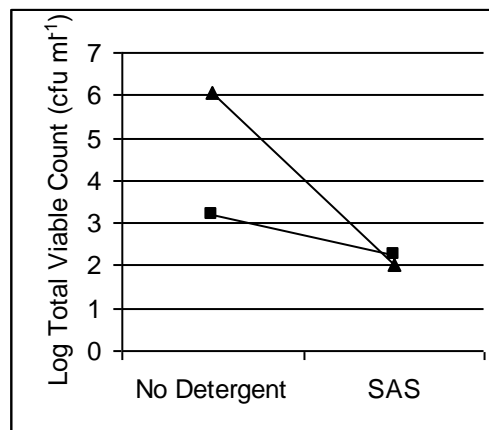


Figure 3.21. Interaction plot of *L. monocytogenes* treated with SAS for 2 hours followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=9$ . SE shown.

Figures 3.20 and 3.21 compare the effect of treating *L. monocytogenes* with SAS for 20 minutes and 2 hours on susceptibility to BAC. In Figure 3.20 it can be seen that the TVC for untreated cells was Log 6.28 which was significantly

reduced to Log 2.22 following treatment with SAS for 20 minutes and to Log 2.94 following treatment with BAC ( $p < 0.01$  for both). Treatment with detergent followed by disinfectant reduced TVC to Log 2.35 and a convergence of the lines shows that *L. monocytogenes* becomes significantly less sensitive ( $p < 0.01$ ) to BAC following treatment with SAS.

In Figure 3.21 it can be seen that the TVC for untreated cells was Log 6.08 which was significantly reduced to Log 2.02 following 2 hours treatment with SAS and to Log 3.20 following treatment with BAC ( $p < 0.01$  for both). Treatment with detergent followed by disinfectant reduced TVC to Log 2.25 and a convergence of the lines shows that *L. monocytogenes* becomes significantly more susceptible ( $p < 0.01$ ) to BAC following treatment with SAS, but no difference in effect was observed as result of longer exposure to detergent.

### 3.2.5 Time course analysis of susceptibility of *L. monocytogenes* treated with FAE up to 2 hours followed by BAC.

Although FAE had no effect on susceptibility of *L. monocytogenes* to BAC following treatment of 20 minutes (Figure 3.6), time of exposure to the detergent was increased to 2 hours to determine whether the non-ionic detergent would have any effect over a longer period of time.

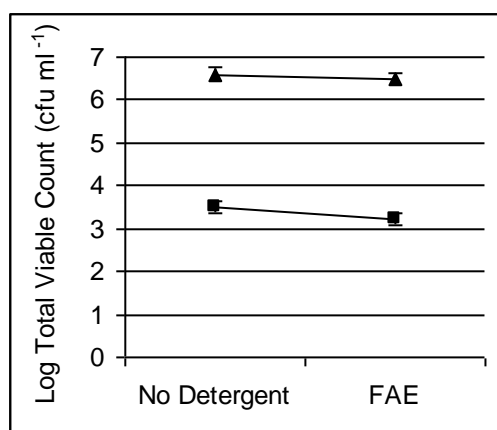


Figure 3.22. Interaction plot of *L. monocytogenes* treated with FAE for 20 minutes followed by BAC. ▲ = no disinfectant, ■ = disinfectant.  $n=18$ . SE shown.

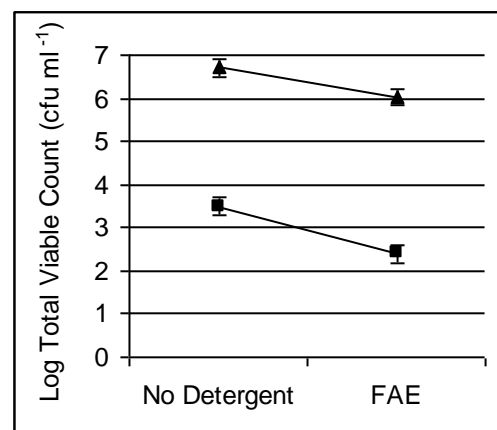


Figure 3.23. Interaction plot of *L. monocytogenes* treated with FAE for 2 hours followed by BAC. ▲ = no disinfectant. 77

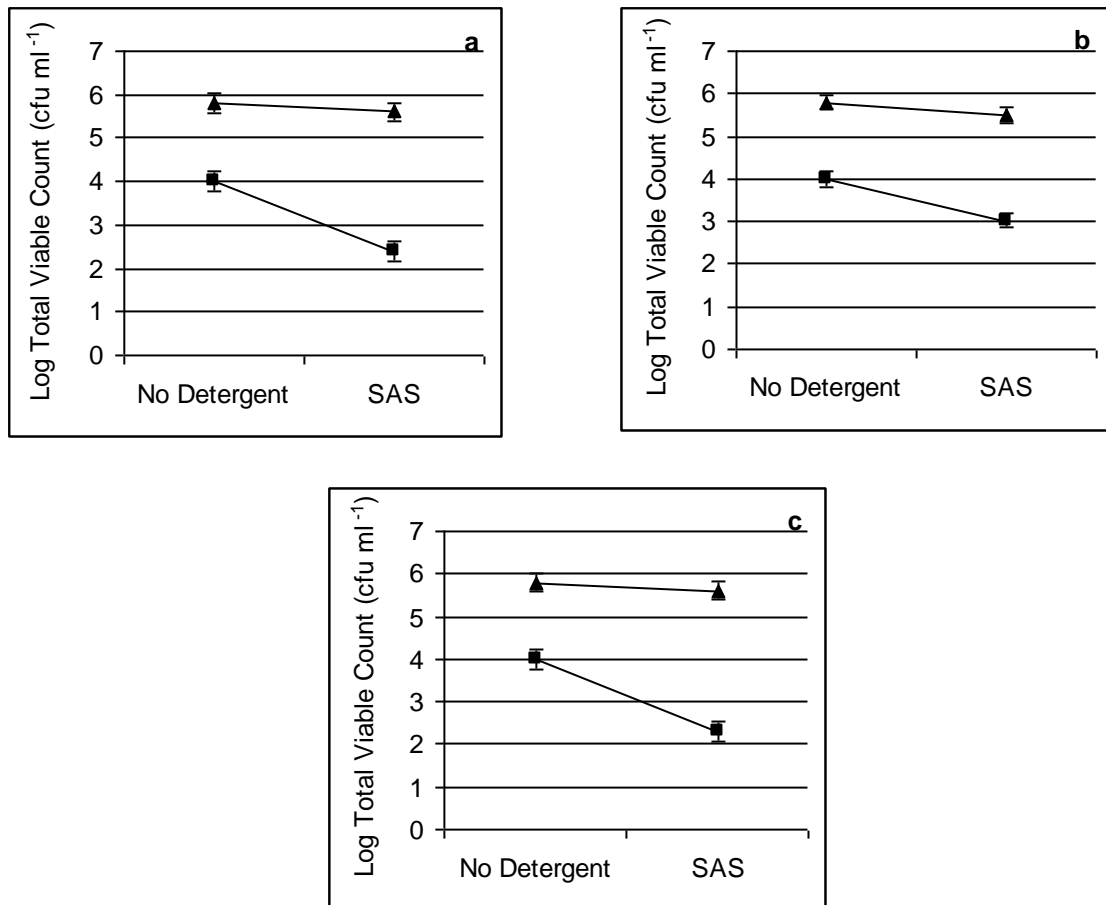
Figures 3.22 and 3.23 compare the effect, on susceptibility to BAC, of treating *L. monocytogenes* with FAE for 20 minutes and 2 hours. In Figure 3.22 it can be seen that the TVC for untreated cells was Log 6.63. Treatment with FAE for 20 minutes had no significant effect on TVC ( $p=0.2$ ) while treatment with BAC alone significantly reduced ( $p<0.01$ ) TVC to Log 3.54 (by 3.09 Log). Treatment with detergent followed by disinfectant reduced TVC to Log 3.22 (by 3.4 Log) but no significant interaction effect was observed with the combination ( $p=0.42$ ).

In Figure 3.23 it can be seen that the TVC for untreated cells was Log 6.71 which was significantly reduced ( $p<0.01$ ) to Log 6.03 (by 0.67 Log) following 2 hours treatment with FAE and to Log 3.54 (by 3.17 Log) following treatment with BAC ( $p<0.01$ ). Treatment with detergent followed by disinfectant reduced TVC to Log 2.39 (by 4.3 Log) but no significant interaction effect was observed with the combination ( $p=0.02$ ) and as before no increased effect was observed as result of longer exposure to detergent.

### **3.3. Effect of different concentrations of detergents on susceptibility of *E. coli* to BAC**

#### **3.3.1. Effect of increase in concentration of SAS on susceptibility of *E. coli* to BAC**

Experiments so far have shown that following exposure to SAS and FAE, *E. coli* becomes more susceptible to BAC. To further investigate the effects of the detergents on the cells, experiments were carried out to observe what effect an increase in concentration of SAS would have on susceptibility of *E. coli* to BAC.



Figures 3.24 a-c. Interaction plots of *E. coli* treated with SAS at (a) 0.2 %, (b) 0.4 % and (c) 0.8 % for 20 minutes followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.24 a-c show the effects of different concentrations of SAS (0.2, 0.4 and 0.8 % respectively), for 20 minutes, on the susceptibility of *E. coli* to BAC. It can be seen that none of the concentrations of detergent reduced the TVC by more than Log 0.3 while BAC significantly reduced TVC in each case ( $p=0$  for all). While there was a significant interaction effect of detergent and disinfectant showing a significant increase in susceptibility to BAC at all concentrations of detergent used ( $p<0.01$ , 0.03 and 0 respectively), none of the concentrations had a greater effect on susceptibility to BAC than another. The results show there would be no benefit in increasing the detergent

concentration, over the range tested, for enhancement of a synergistic effect with BAC but, higher concentrations may further increase susceptibility

### 3.3.2 Effect of increase in concentration of FAE on susceptibility of *E. coli* to BAC.

As *E. coli* also becomes more susceptible to BAC following treatment with FAE, experiments were also carried out to observe the effects of an increase in concentration of the detergent.

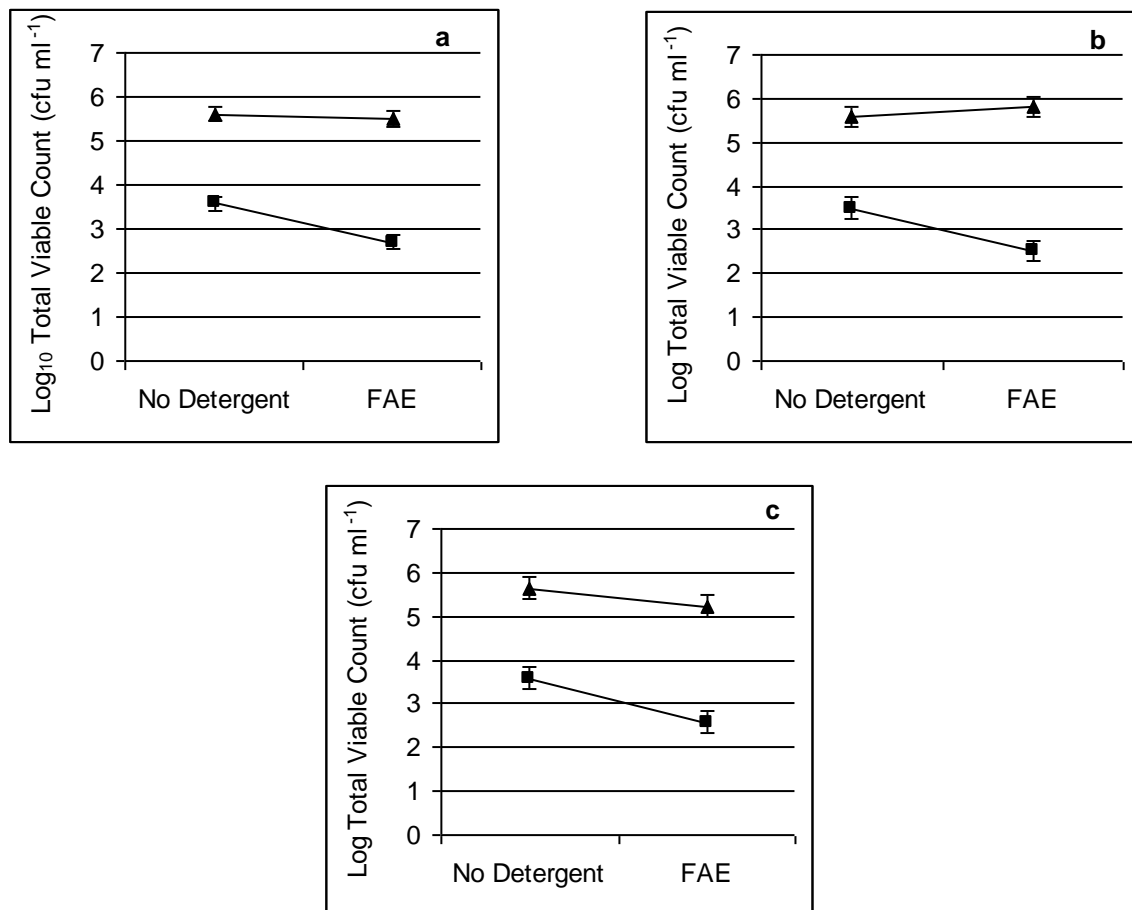


Figure 3.25 a-c. Interaction plots of *E. coli* treated with FAE at (a) 0.1 %, (b) 0.2 % and (c) 0.4 % for 20 minutes followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.



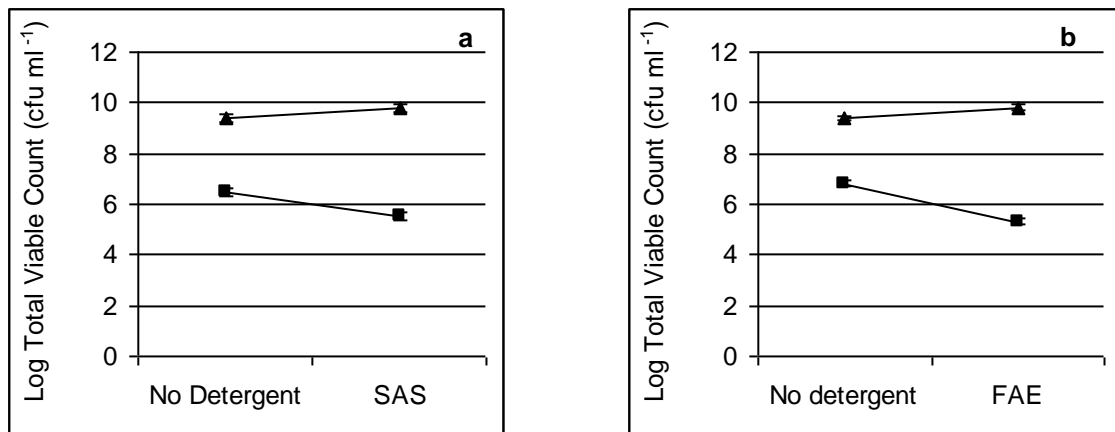
Figures 3.25 a-c show the effects of different concentrations of FAE (0.1, 0.2 and 0.4 % respectively) on the susceptibility of *E. coli* to BAC. It can be seen that only 0.4 % FAE reduced TVC significantly ( $p < 0.01$ ) while BAC reduced TVC significantly for all ( $p < 0.01$ ). An interaction effect was observed following 0.1% FAE ( $p < 0.01$ ), which was seen previously (Figure 3.6), and 0.2% FAE ( $p = 0.01$ ) with a plot divergence showing that *E. coli* becomes significantly more susceptible to BAC however, there was no significant difference in change in susceptibility between the two concentrations.

There was an interaction effect of FAE at 0.4% and BAC, which reduced TVC by more than the sum of the individual effects however, due to the reduction in TVC by the FAE alone, the interaction was not significant ( $p = 0.2$ )

#### **3.4 Effect of detergents on susceptibility of suspended *E. coli* and *L. monocytogenes* to BAC to compare to attached cells.**

Observations of the susceptibility of organisms to disinfectants were performed on attached cells but, due to the nature of other investigations, suspended cells were required. To establish whether the results would correlate, susceptibility testing was performed with suspended cells and the results compared to those of attached cells.

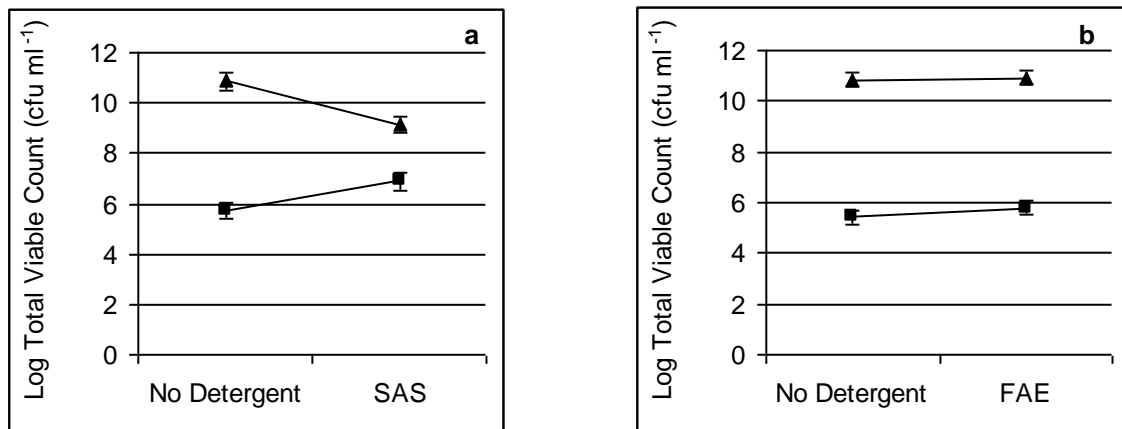
### 3.4.1 *E. coli* treated with SAS and FAE followed by BAC.



Figures 3.26 a-b. Interaction plot of suspended *E. coli* treated with (a) SAS or (b) FAE followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.26 a-b show the effects of treating suspended *E. coli* with the detergents SAS and FAE for 20 minutes followed by BAC for 5 minutes. It can be seen that the TVC for untreated cells was Log 9.42 which increased to Log 9.85 and Log 9.87 following treatment with SAS and FAE respectively which may be due to growth of cells or disruption of cell clusters by the detergents. BAC alone significantly reduced ( $p < 0.01$ ) TVC to Log 6.55 and Log 6.8 respectively. Treatment with detergent followed by disinfectant significantly reduced ( $p < 0.01$ ) TVC to Log 5.5 (by 3.92 Log) and Log 5.34 (by 4.08 Log) respectively and the effect of the interaction gives a plot divergence that shows that suspended *E. coli* becomes significantly more susceptible to BAC following treatment with both detergents.

### 3.4.2 *L. monocytogenes* treated with SAS and FAE followed by BAC.



Figures 3.27 a-b. Interaction plot of suspended *L. monocytogenes* treated with (a) SAS or (b) FAE followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=27. SE shown.

Figures 3.27 a-b show the effects of treating suspended *L. monocytogenes* with the detergents SAS and FAE for 20 minutes followed by BAC for 5 minutes. It can be seen that the TVC for untreated cells was Log 10.85 which was reduced to Log 9.14 (by 1.71 Log) with SAS and increased to Log 10.92 with FAE respectively while BAC alone significantly reduced TVC ( $p < 0.01$ ). Compared to attached cells the TVC for untreated cells was approximately 4 Log orders higher however, despite the high numbers, the extent of the effect of the detergent and disinfectant combinations was similar. Treatment with SAS followed by BAC significantly reduced ( $p < 0.01$ ) TVC to Log 6.88 (by 3.97 Log) which for attached cells was reduced by 4.06 Log. The plot convergence shows the same trend as attached cells with suspended *L. monocytogenes* becoming significantly more resistant to BAC following treatment with SAS.

FAE followed by BAC significantly reduced TVC ( $p < 0.01$ ) to Log 5.8 (5.05 Log) which for attached cells was reduced by 3.5 Log and, as with attached

cells, there was no interaction between the detergent and disinfectant that had any effect on susceptibility ( $p=0.3$ ).

As the TVC of suspended cells was much higher than attached cells, it may be expected that detergent, at the same concentration, would have less effect. However, suspended cells have a greater surface area exposed to the effects of the detergent than cells attached to surfaces which resulted in the same trends and comparable reductions in TVC. This enabled further experiments to be performed with cells in suspension.

### **3.5. Investigations into the mechanisms of detergent induced changes in disinfectant susceptibility.**

It has been established that treating *E. coli* and *L. monocytogenes* with different detergents affects the susceptibility of the cell to disinfectants. The aim now was to investigate why these changes in susceptibility were occurring.

#### **3.5.1. Assessment by flow cytometry of the effect of detergents on cell membrane permeability of suspended cells over time.**

One possible explanation for the changes seen in susceptibility of the organisms to disinfectant may be effects of the detergents on membrane permeability. Previous experiments have shown that when treated with detergent for different times, changes can occur in susceptibility so experiments were carried out at different times to determine whether longer exposure would cause changes in permeability that could be related to susceptibility. Suspended cells were treated with the detergents for 20

minutes and 2 hours and were washed in Ringers solution to remove non-bound detergent from the surface. The fluorescent dye PI was added to samples of the cells and changes in fluorescence, that indicated alterations in cell membrane permeability, were observed by flow cytometric analysis.

### 3.5.2. Effect of time of exposure to detergent on cell membrane permeability of *E. coli*.

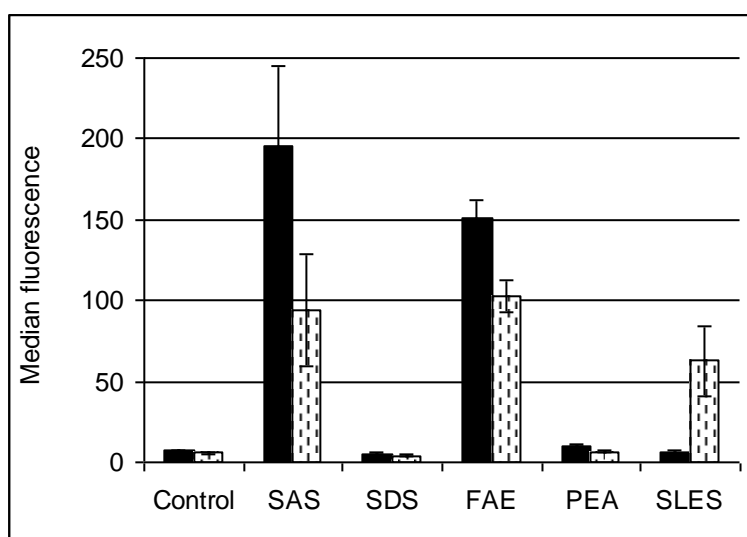


Figure 3.28. Effect on cell membrane permeability of *E. coli* treated with detergents for 20 minutes and 2 hours as determined by flow cytometric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. ■ = 20 minutes. ▨ = 2 hours. n=9. SE shown.

Figure 3.28 shows the effect of detergents on cell membrane permeability of *E. coli* with an increase in permeability of the cell envelope indicated by enhanced uptake of PI to give fluorescence. Following 20 minutes treatment with SAS and FAE, fluorescence increased by greater than 20 times, compared to untreated control cells and although still significant, this reduced following 2 hours treatment to more than 15 times ( $p < 0.01$  for all). SDS had no effect on permeability at either time ( $p = 0.08$  and  $0.6$ ). There was a significant increase in fluorescence following 20 minutes treatment with PEA

( $p < 0.01$ ) but no effect was observed following 2 hours treatment ( $p = 0.14$ ) and following treatment with SLES, no change was observed after 20 minutes ( $p = 0.59$ ) but a significant increase in fluorescence was observed after 2 hours ( $p < 0.01$ ).

### 3.5.3. Effect of time of exposure to detergent on cell membrane permeability of *L. monocytogenes*

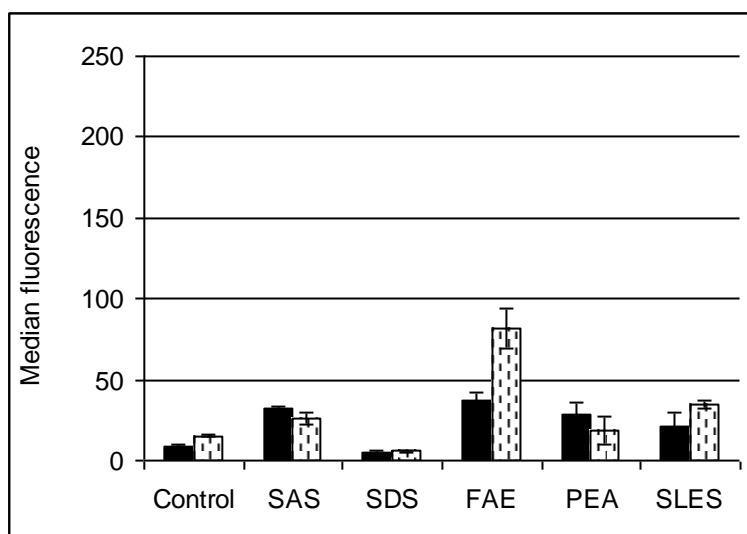


Figure 3.29. Effect on cell membrane permeability of *L. monocytogenes* treated with detergents for 20 minutes and 2 hours as determined by flow cytometric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. ■ = 20 minutes, ▨ = 2 hours.  $n = 9$ . SE shown.

Figure 3.29 shows the effect of detergents on cell membrane permeability of *L. monocytogenes*. Compared to untreated control cells, fluorescence significantly increased by 2-4 times following 20 minutes treatment with SAS, FAE, PEA and SLES ( $p < 0.01$  for all) but there was no difference observed between the detergents and no change was observed following treatment with SDS ( $p = 0.2$ ). Following 2 hours treatment with SAS there was still a significant increase in fluorescence although less than at 20 minutes, and SDS still had no effect ( $p = 0.1$ ). Compared to control cells, there was a further

significant increase in fluorescence of 3-5 times following 2 hours treatment with FAE and SLES while there was no significant difference with cells treated with PEA ( $p=0.1$ ).

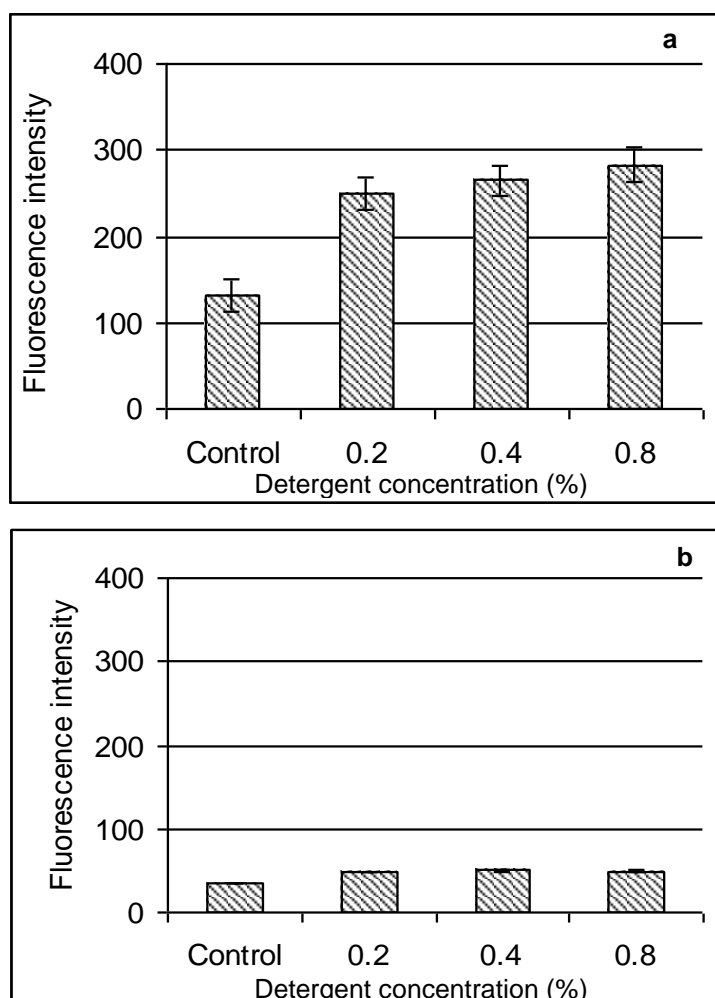
Table 3.2. Summary of effect of exposure to detergents for different times on cell membrane permeability of *E. coli* and *L. monocytogenes*. + = small / some change, ++ = large change, O = no change.

<i>E.coli</i>	SAS	SDS	FAE	PEA	SLES
20 minutes	++	O	++	+	O
2 hours	+	O	+	O	+
<i>L.monocytogenes</i>					
20 minutes	+	O	+	+	+
2 hours	+	O	++	+	+

For practicality purposes it was decided to continue experiments using the fluorimeter rather than the flow cytometer and test samples run through both pieces of equipment confirmed that the results showed the same trend (results not shown).

### 3.6. Increase in concentration of detergent on cell membrane permeability assessed with the fluorimeter.

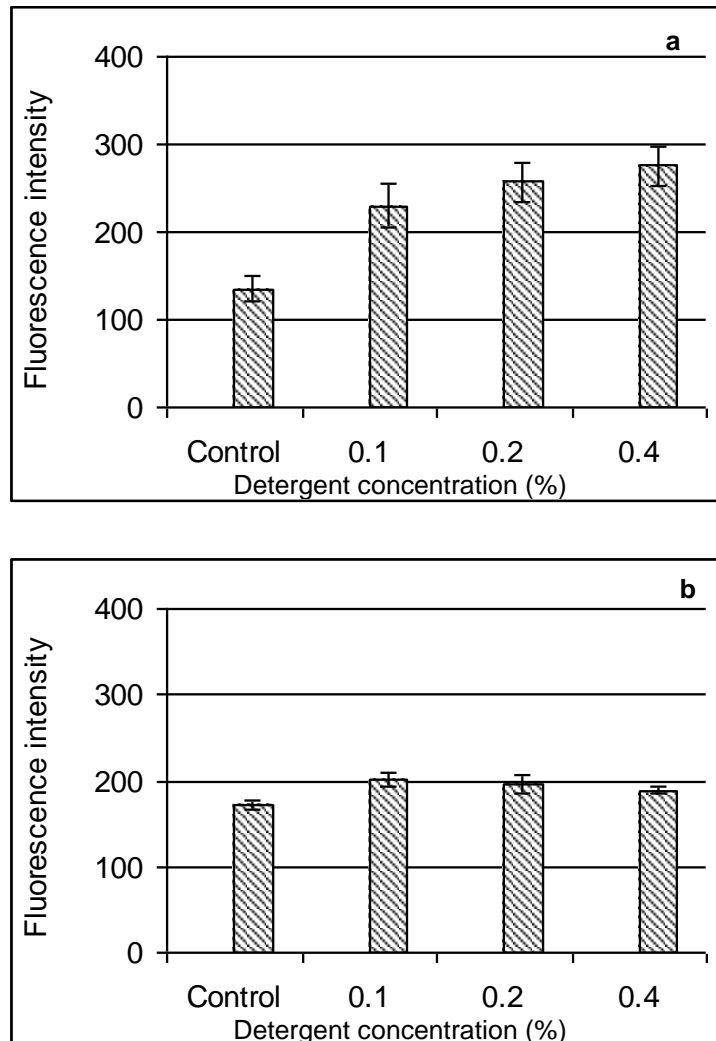
Observations have shown that SAS and FAE, which are detergents commonly used in food industry cleaning procedures, cause significant increases in fluorescence over time (Figures 3.28 and 3.29) suggesting increases in cell membrane permeability. To follow this *E. coli* and *L. monocytogenes* were treated to these detergents at increasing concentrations to determine whether this would also have an effect on cell membrane permeability.



Figures 3.30 a and b. Effect of increase in concentration of SAS on cell membrane permeability of (a) *E. coli* and (b) *L. monocytogenes* as determined by fluorimetric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. n=9. SE shown.

Figures 3.30 a and b show that an increase in concentration of SAS from 0.2 % to 0.8 % causes a statistically significant increase in fluorescence of both *E. coli* (2 fold) and *L. monocytogenes* (0.3 fold) to all concentrations compared to the control cells ( $p < 0.01$  for all), with the extent of the effect being markedly higher with *E. coli*. However, there is no significant difference in effect on cell membrane permeability between each of the concentrations used, for either of the detergents ( $p > 0.05$  for all).



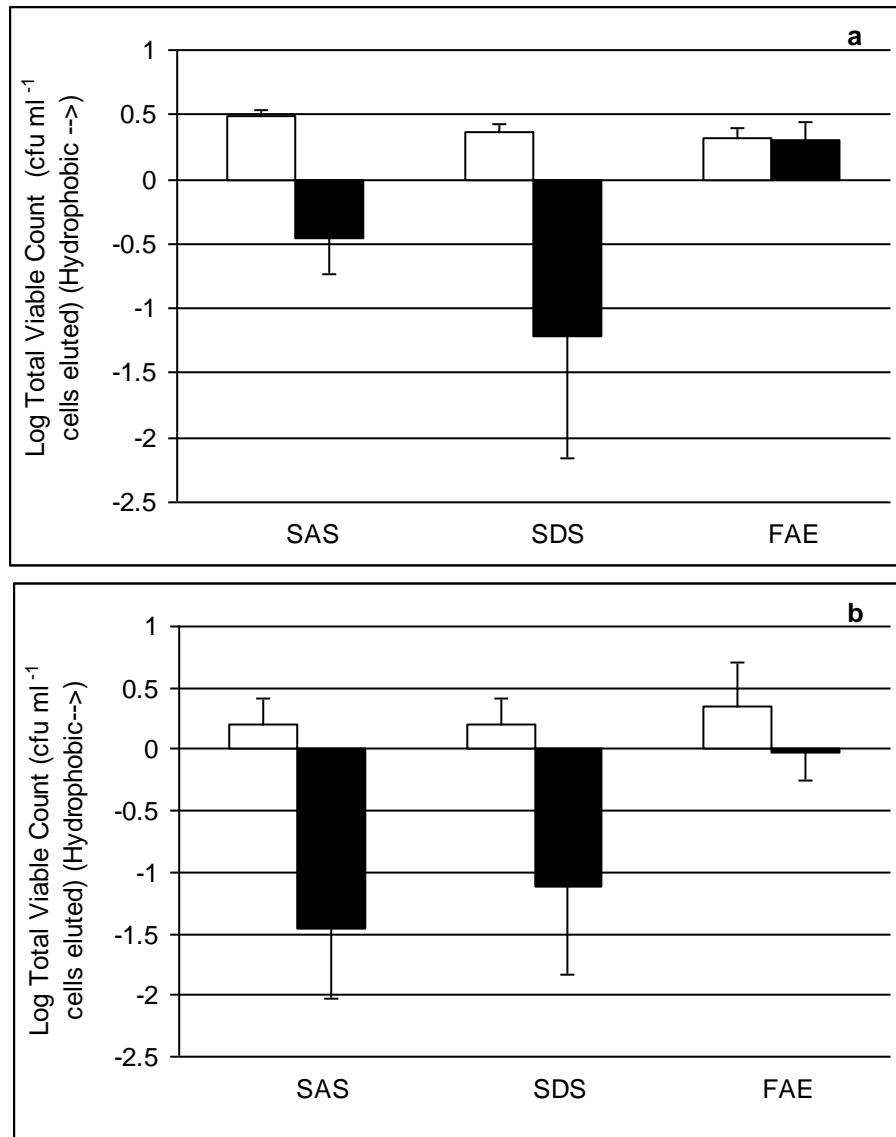


Figures 3.31 a and b. Effect of increase in concentration of FAE on cell membrane permeability of (a) *E. coli* and (b) *L. monocytogenes* as determined by fluorimetric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. n=9. SE shown.

Figures 3.31 a and b show that an increase in concentration of FAE causes a significant increase in fluorescence of both *E. coli* (2 fold) and *L. monocytogenes* (0.3 fold) to all concentrations compared to the control cells ( $p < 0.01$  for all) with the extent of the effect being greater with *E. coli*. As before there is no difference in effect on cell membrane permeability between each of the concentrations of detergent used ( $p > 0.05$  for all).

### **3.7. Effects of detergents on cell surface hydrophobicity**

Hydrophobicity is important as changes brought about by a detergent binding to the surface, can affect the approach and interaction of a disinfectant. Experiments were carried out to determine whether detergents caused changes in cell surface properties that may affect susceptibility to disinfectants. Suspended cells were exposed to detergents for 20 minutes then passed through sepharose and octyl sepharose columns. Through analysis of retention, changes in hydrophobicity were determined by calculating the  $\log_{10}$  difference in TVC of untreated and treated cells eluted from the sepharose and octyl sepharose columns (Clark *et al.*, 1985).



Figures 3.32 a and b. Change in hydrophobicity of (a) *E. coli* and (b) *L. monocytogenes* following treatment with detergents. □ = cells eluted from the sepharose column, ■ = cells eluted from the octyl sepharose column. n=12. SE shown.

Figure 3.32a shows that *E. coli* becomes significantly more hydrophilic ( $p < 0.01$ ) after treatment with SAS and SDS but no change was observed in cells treated with FAE. There was also a significant difference between the effect of the detergents with those treated with SDS being significantly more hydrophilic ( $p < 0.01$ ) than those treated with SAS.

*L. monocytogenes* becomes significantly ( $p < 0.01$ ) more hydrophilic after treatment with all of the detergents and there was no significant difference between the effects of the two anionic detergents.

### **3.8. Efflux experiments**

#### **3.8.1. Determination of cell and EtBr volumes for efflux experiments.**

Prior to investigations into a possible efflux mechanism in *L. monocytogenes*, experiments were carried out to establish the most suitable combinations of cells and EtBr that would give measurable uptake and efflux. Cells were prepared, as described in the methods, by being depleted of energy that may drive efflux through several centrifugation steps in ice-cold HT buffer. Different volumes of cells were combined with 0.04 ml EtBr, which had previously been determined to be an appropriate volume to use (results not shown) and uptake of EtBr was observed until cells were fully loaded at 50 minutes. Energy was provided back to the cells through the addition of glucose and efflux of EtBr was observed by fluorimetric analysis.

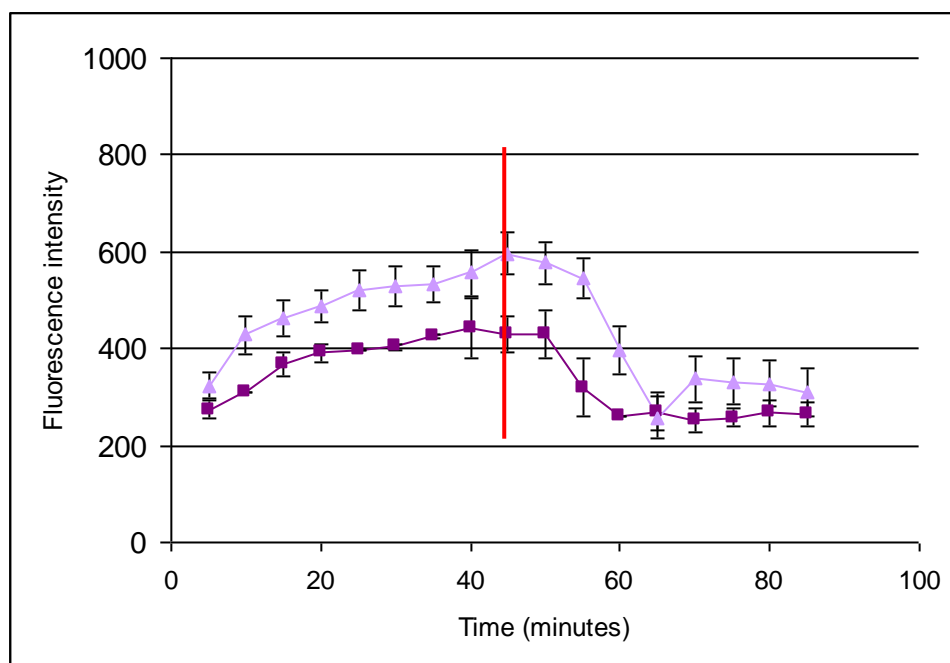


Figure 3.33. Uptake and efflux of EtBr by *L. monocytogenes* using different volumes of cells and 0.04 ml EtBr as determined by fluorimetric analysis, with an excitation wavelength of 510 nm and an emission wavelength of 595 nm. ■ = 0.1 ml cells + 0.04 ml EtBr, ▲ = 0.2 ml cells + 0.04 ml EtBr. | = addition of glucose. n=3. SE shown.

Figure 3.33 shows that the combination that gave the most measurable uptake and efflux of EtBr was 0.2 ml of prepared cells with 40  $\mu$ l of EtBr and this would be used for further efflux experiments. The results show that EtBr is removed from *L. monocytogenes* following the addition of glucose, which strongly suggests the presence of an efflux mechanism.

### 3.8.2. Effect of temperature on efflux experiments

Investigations into efflux by Midgley (1986) and Aase *et al.* (2000) were performed at 37 °C however, as all previous experiments in this study were performed at 20 °C, it needed to be established whether the effect of temperature would influence uptake and efflux of EtBr. Cells were prepared as described as previously and were maintained at 20 °C or 37 °C for the duration of the experiments. Uptake was recorded until cells became fully

loaded with EtBr then glucose was added and efflux was observed by fluorimetric analysis.

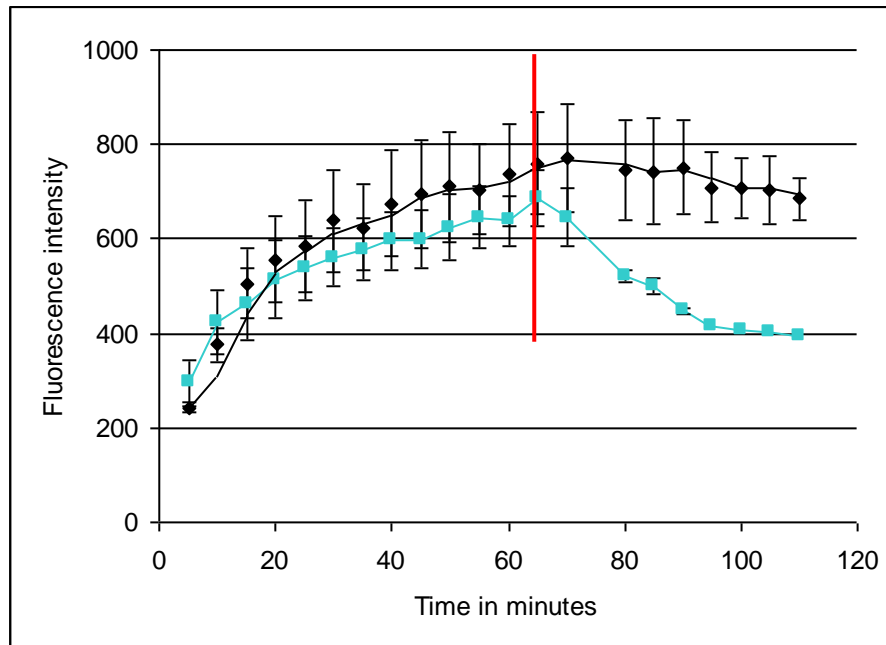


Figure 3.34a. Uptake and efflux of EtBr by *L. monocytogenes* at 20 °C as determined by fluorimetric analysis with an excitation wavelength of 510 nm and an emission wavelength of 595 nm. ● = no glucose added, ■ = glucose added. | = addition of glucose. n=3. SE shown.

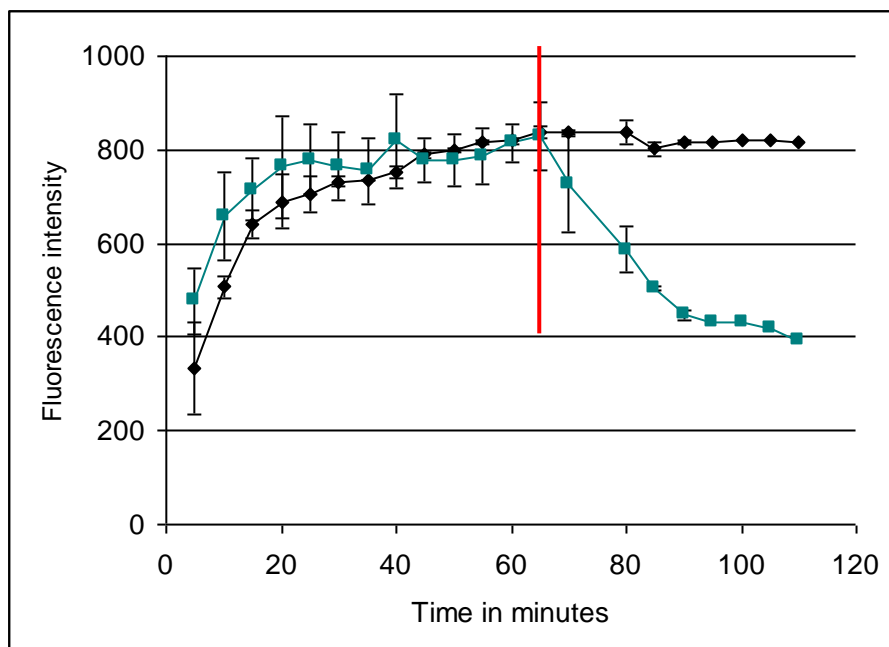


Figure 3.34b. Uptake and efflux of EtBr by *L. monocytogenes* at 37 °C as determined by fluorimetric analysis with an excitation wavelength of 510 nm and an emission wavelength of 595 nm. ● = no glucose added, ■ = glucose added. | = addition of glucose. n=3. SE shown.

Figures 3.34 a and b show no difference in the uptake of EtBr at 20 °C and 37 °C up to 60 minutes although the rate of efflux of EtBr from cells maintained at 37 °C is significantly more rapid ( $p=0.04$ ) than those maintained at 20 °C. As the same trend was observed at both temperatures, and uptake and efflux were measurable, further experiments were continued at 20 °C to maintain consistency throughout the work.

### 3.8.3. Efflux of EtBr from *L. monocytogenes* using glucose and detergents.

Once optimum conditions had been established, experiments were carried out to investigate whether the detergents would have any effect on efflux of EtBr from *L. monocytogenes*. Cells were prepared for efflux experiments as described previously and uptake of EtBr was observed until the cells were fully loaded. Glucose was then added either on its own or in combination with SAS or FAE and efflux was observed by fluorimetric analysis.

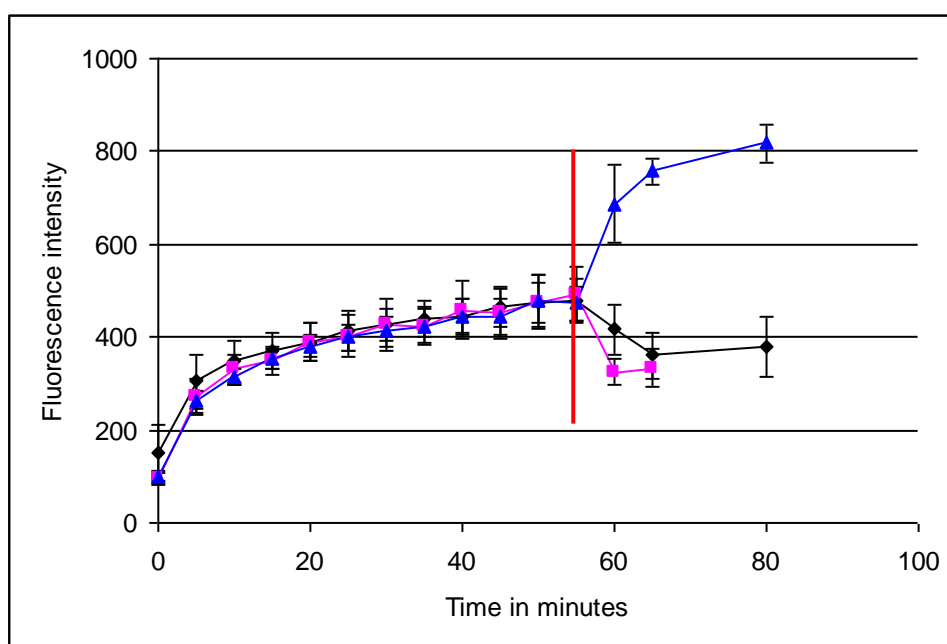


Figure 3.35. Uptake and efflux of EtBr by *L. monocytogenes* as determined by fluorimetric analysis with an excitation wavelength was 510 nm and an emission wavelength of 595 nm following addition of; ♦ = glucose, ■ = glucose and SAS, ▲ = glucose and FAE. | = addition of glucose.  $n=12$ . SE shown.

It can be seen in figure 3.35 that the uptake of EtBr was the same for all samples but following addition of glucose or glucose and detergent, differences in the rate of efflux occur. Although not significant ( $p=0.3$ ), the rate of efflux from cells with glucose and SAS added in combination appeared to be more rapid than those with glucose added alone. When FAE and glucose were added to the cells there was a significant increased uptake of EtBr, which requires further investigation.

#### 3.8.4. Efflux of EtBr by *L. monocytogenes* pre-exposed to detergent before uptake and efflux

In Figure 3.35 it can be seen that the addition of detergents had an effect on the efflux of EtBr from *L. monocytogenes* so investigations continued into how detergents may affect uptake of EtBr into the cells. Cells were pre exposed to a water control, SAS or FAE for 20 minutes before being prepared for the efflux experiments as previously. Uptake of EtBr was observed until the cells were fully loaded before the addition of glucose, glucose and SAS combined or glucose and FAE combined. Efflux was observed by fluorimetric analysis.

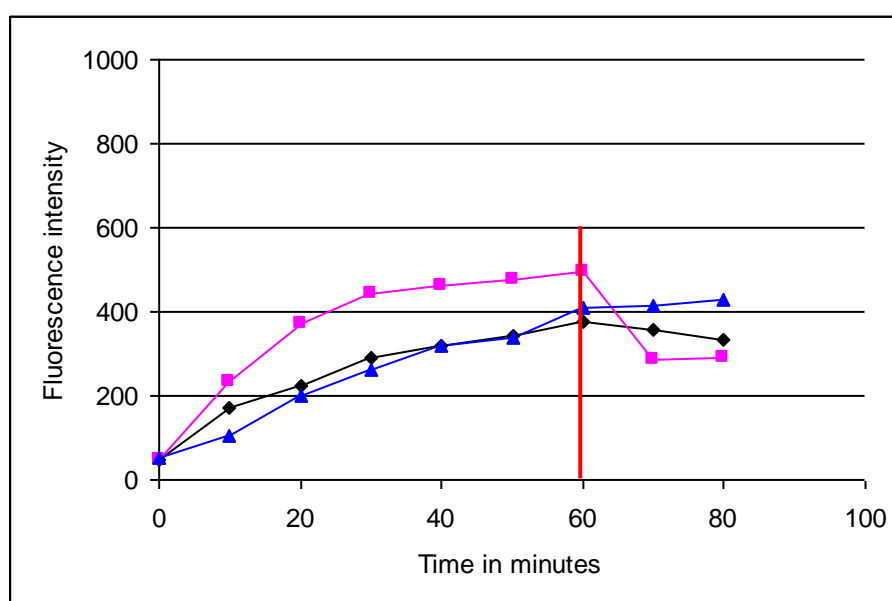


Figure 3.36. Uptake of EtBr by *L. monocytogenes* pre treated with water (♦), SAS (■) or FAE (▲), and efflux following addition of glucose only (♦), glucose and SAS (■) or glucose and FAE (▲) as determined by fluorimetric analysis with an excitation wavelength was 510 nm and an emission wavelength of 595 nm. | = glucose added. n=1.



Although this was a preliminary study and only carried out once, the results show that there is no difference in the uptake of EtBr between the control cells and those pretreated with FAE but there is a significant increase in the uptake by cells that were pre treated with SAS. On addition of glucose alone or in combination with the detergents, the same response is seen as in figure 3.35 where the rate of efflux is the most rapid when glucose was added in combination with SAS and uptake of EtBr continued for cells were glucose was added in combination with FAE.

#### **3.8.5. Effect of SAS or FAE on permeability of *L. monocytogenes* over time**

Figure 3.36 showed that pre treatment of *L. monocytogenes* with SAS led to a significant increase in the uptake of EtBr and a continued uptake when pre treated with FAE. To try and understand why this was occurring, a time course analysis was carried out to determine whether this might be related to cell membrane permeability. For comparison, suspended cells were exposed to SAS or FAE for different times and were washed in Ringers solution to remove excess detergent from the surface. PI was added to samples of the cells and fluorescence was observed by flow cytometry.

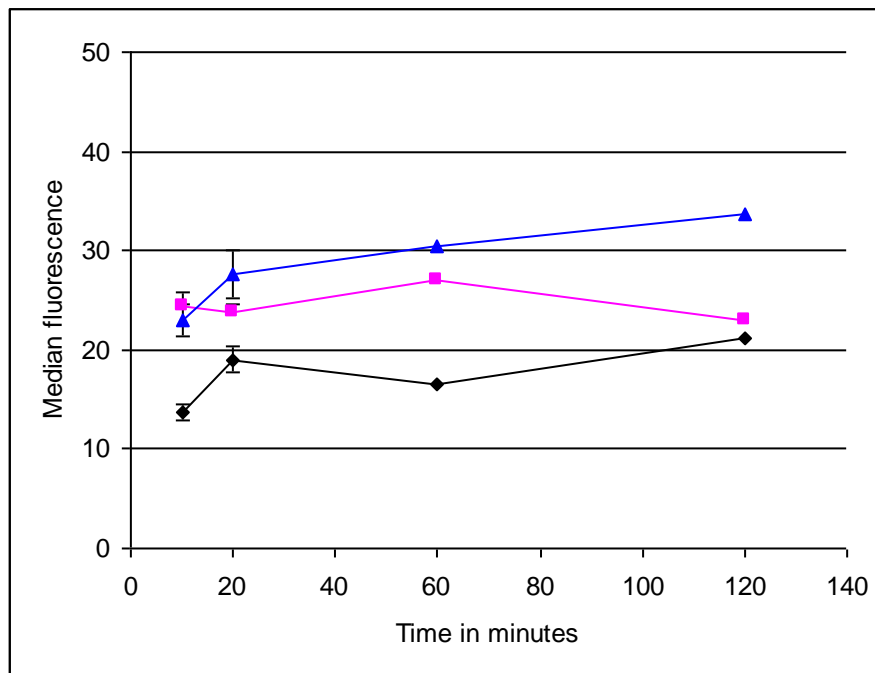
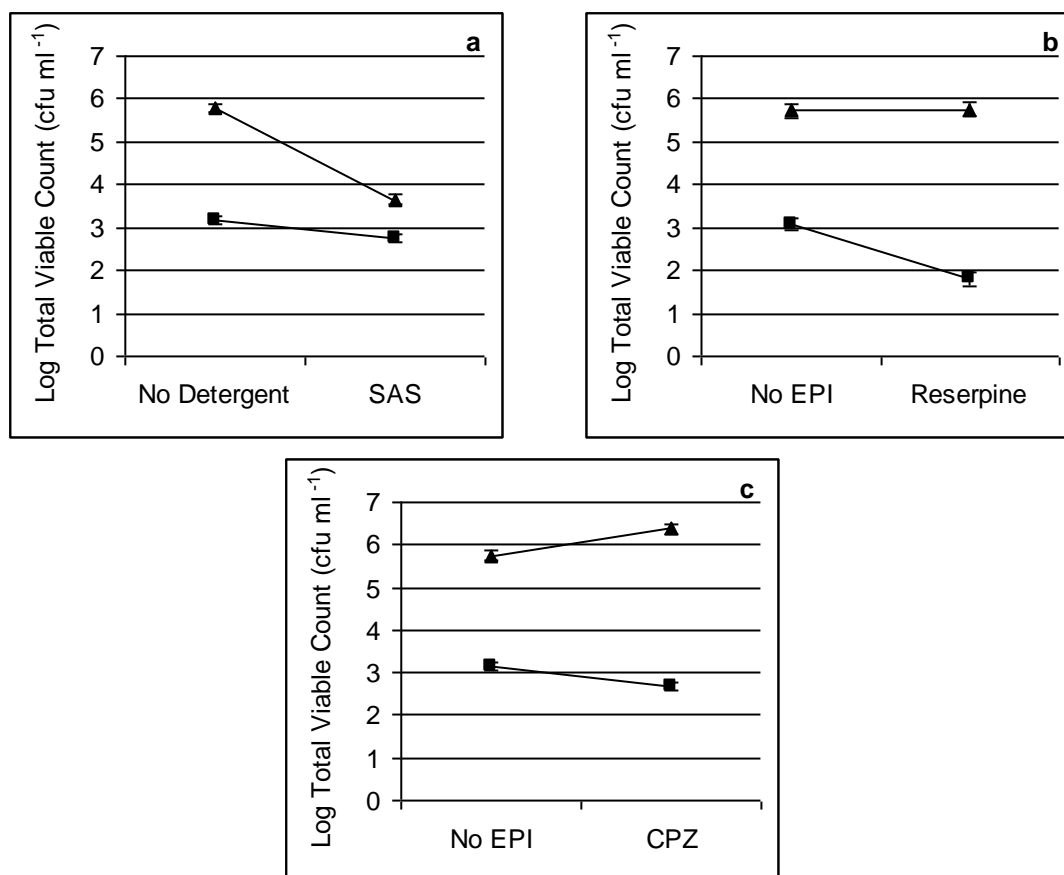


Figure 3.37. Effect of SAS or FAE on permeability of suspended *L. monocytogenes* over time as determined by flow cytometric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. ♦ = Control, ■ = SAS, ▲ = FAE. n=3. SE shown.

It can be seen in figure 3.37 that compared to the control, the cell membrane of *L. monocytogenes* becomes significantly more permeable ( $p < 0.01$ ) following 5 minutes treatment with both detergents and the difference is greater for cells treated with FAE. Over time there is no significant difference that suggests pre treatment with SAS leads to the greater increase in uptake of EtBr. The results agree with Figure 3.29 where following 2 hours treatment with SAS there was no significant change in cell membrane permeability but following 2 hours treatment with FAE there is a significant increase in cell membrane permeability.

### **3.8.6. Effect of efflux pump inhibitors (EPIs) on susceptibility of *L. monocytogenes* to BAC.**

Although observations have so far shown that EtBr is removed from *L. monocytogenes* on the addition of glucose, it needed to be confirmed whether this was due to the presence of an efflux pump. Efflux pump inhibitors (EPIs) such as chlorpromazine (CPZ) or reserpine are inhibitors of metabolic processes in the cell that are essential for efflux pump activity and can be used to determine if an efflux pump is active in an organism. However, before continuing with experiments to determine whether the EPIs would inhibit efflux, experiments were carried out to establish whether they had any effect of TVC and if they affected susceptibility of the cells to disinfectant. Attached cells were treated with SAS, CPZ or reserpine for 20 minutes, rinsed in Ringers solution to remove unattached cells, and then treated in BAC for 5 minutes. The surfaces were swabbed and TVC was determined.

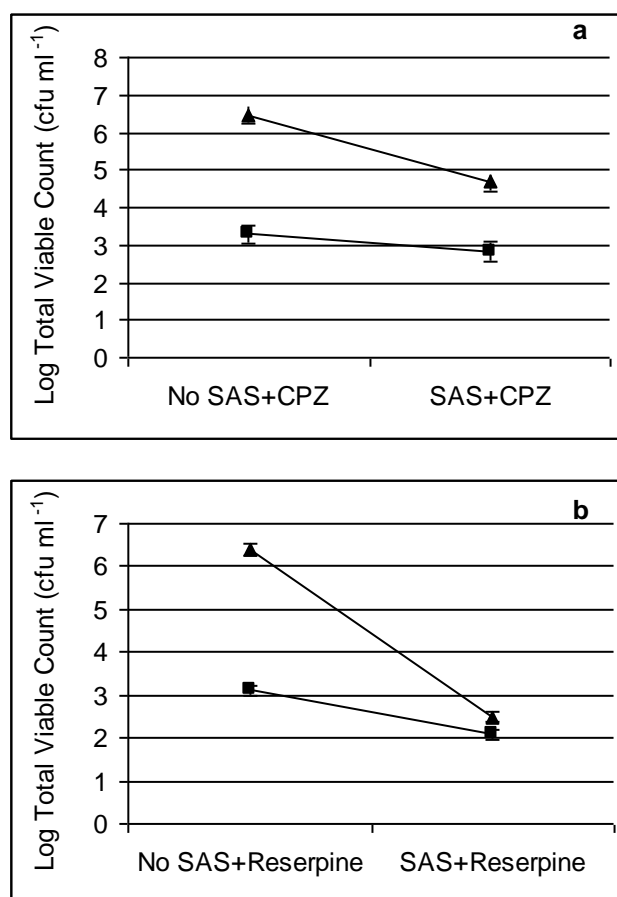


Figures 3.38 a-c. Effect of 20 minutes treatment of (a) SAS, (b) CPZ and (c) reserpine on susceptibility of *L. monocytogenes* to BAC. ▲ = no disinfectant, ■ = disinfectant. n=15. SE shown.

It can be seen in figure 3.38a that SAS causes a significant reduction ( $p < 0.01$ ) in TVC of *L. monocytogenes* while the EPIs (3.38b and c) have no effect and BAC significantly reduced TVC by an average of Log 2.6 ( $p < 0.01$  for all). In combination SAS and BAC reduced TVC to Log 2.76 (by 3.02 Log), CPZ and BAC reduced TVC to Log 2.68 (by 3.07 Log) and reserpine and BAC reduced TVC to Log 1.8 (by 3.94 Log). In Figure 3.38a plot convergence shows that *L. monocytogenes* becomes significantly less susceptible to BAC following treatment with SAS while Figures 3.38b and c show by plot divergence that *L. monocytogenes* become significantly more susceptible to BAC following treatment with CPZ and reserpine suggesting that an efflux mechanism may have been inhibited.

### 3.8.7. Effect of EPIs in combination with SAS on susceptibility of *L. monocytogenes* to BAC.

It is thought that the changes in susceptibility to BAC, following detergent exposure in *L. monocytogenes*, may be due to an efflux mechanism induced by SAS so experiments were carried out to determine whether the EPIs had any effect on the action of the detergent. Cells were prepared as for previous susceptibility experiments and the cells were treated for 20 minutes with SAS in combination with CPZ or SAS in combination with reserpine. The surfaces were rinsed in Ringers solution to remove unattached cells, and then treated in BAC for 5 minutes. The surfaces were swabbed and TVC was determined.



Figures 3.39 a-b. Effect of (a) SAS and CPZ and (b) SAS and reserpine on susceptibility of *L. monocytogenes* to BAC. ▲ = no disinfectant, ■ = disinfectant. n=15. SE shown.

The results in figures 3.39a and b show that following treatment with SAS plus CPZ or SAS plus reserpine, there is a significant reduction in susceptibility to BAC ( $p=0$  for all), which is the same as when treated with SAS alone (Figure 3.2). While figures 3.38 b and c suggests that the EPIs have inhibited an efflux mechanism, this result suggests that SAS overrides the effect of the EPIs and efflux was maintained.

### 3.8.8. Effect of CPZ on cell membrane permeability of *L. monocytogenes*

CPZ is an EPI that disrupts metabolic processes by limiting proton production that is required to maintain the proton motive force that drives the efflux pumps (Viveiros *et al.*, 2008). Before continuing with investigations into efflux with *L. monocytogenes*, the effect of CPZ on cell membrane permeability was evaluated. The cells were treated with SAS, CPZ or a combination of both for 20 minutes and were washed in Ringers solution to remove detergent or EPI that was not bound to the cell the surface. PI was added to samples of the cells and fluorescence was observed.

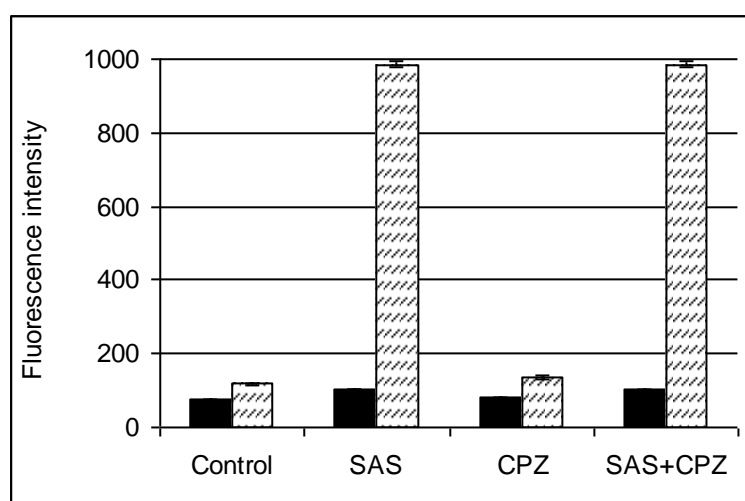


Figure 3.40. A comparison of the effects of SAS, CPZ and SAS with CPZ on cell membrane permeability of *L. monocytogenes* by flow cytometric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. ■ = no PI, ▨ = PI.  $n=9$ . SE shown.

The results in figure 3.40 show that SAS significantly ( $p < 0.01$ ) increased the cell membrane permeability of *L. monocytogenes* compared to the water control while CPZ did not have any significant effect. SAS in combination with CPZ had the same significant effect as SAS alone suggesting that the observed increase in cell membrane permeability was due to the effect of the SAS and not CPZ

### 3.8.9. Effect of EPIs on uptake of ethidium bromide by *L. monocytogenes*

In figure 38 a-c it was seen that following treatment with the EPIs, *L. monocytogenes* becomes more susceptible to BAC. To further confirm this was due to the EPIs suppressing an efflux mechanism, CPZ was incorporated into the experiments investigating efflux. Cells were prepared for efflux experiments as previous and uptake of EtBr was observed until cells were fully loaded. Glucose alone, glucose with SAS, glucose with CPZ and glucose with SAS and CPZ was added to the cells and efflux was observed by fluorimetric analysis.

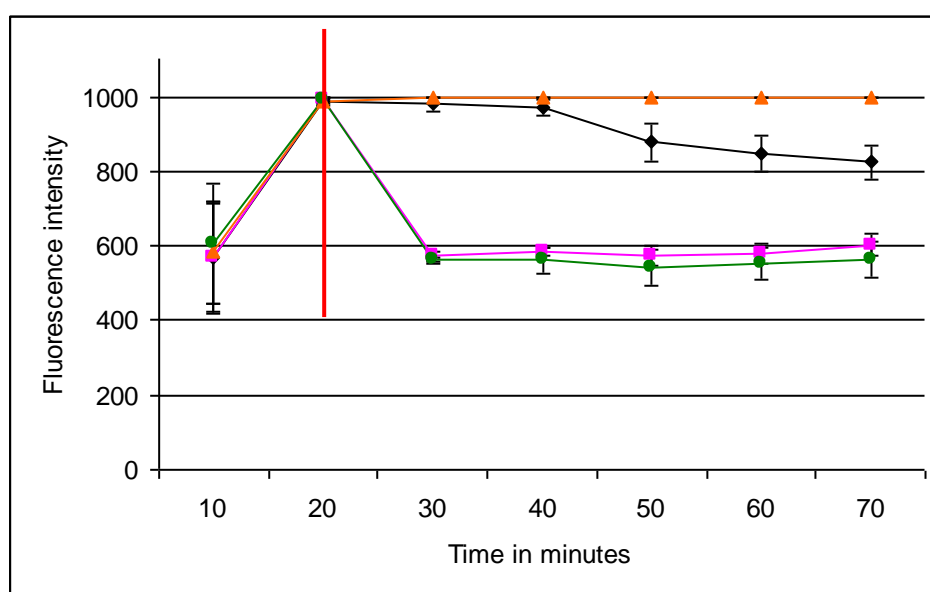


Figure 3.41. Uptake of EtBr by *L. monocytogenes* and efflux following addition of; ♦ = glucose, ▲ = glucose and CPZ, ■ = glucose and SAS, ● = glucose, SAS and CPZ as determined by fluorimeter. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm. | = addition of glucose. n=3. SE shown.

Figure 3.41 shows a steady of rate efflux of EtBr from the cells following the addition of glucose alone but no efflux at all was observed following glucose in combination with CPZ. A rapid rate of efflux was observed on addition of glucose in combination with SAS and glucose in combination with SAS and CPZ but there was no significant difference between the two ( $p=0.8$ ). As before, the results suggest that CPZ was able to switch off an efflux mechanism when in combination with glucose but, when added with SAS, the detergent had a gross effect that counteracted the effect of CPZ and efflux was maintained.

### 3.8.10. Uptake and efflux of EtBr by *L. monocytogenes* and effect of reserpine

The previous experiment was repeated using the EPI reserpine. Uptake of EtBr was observed until cells were fully loaded and glucose alone, glucose with SAS, glucose with reserpine and glucose with SAS and reserpine was added to the cells and efflux was observed by fluorimetric analysis.

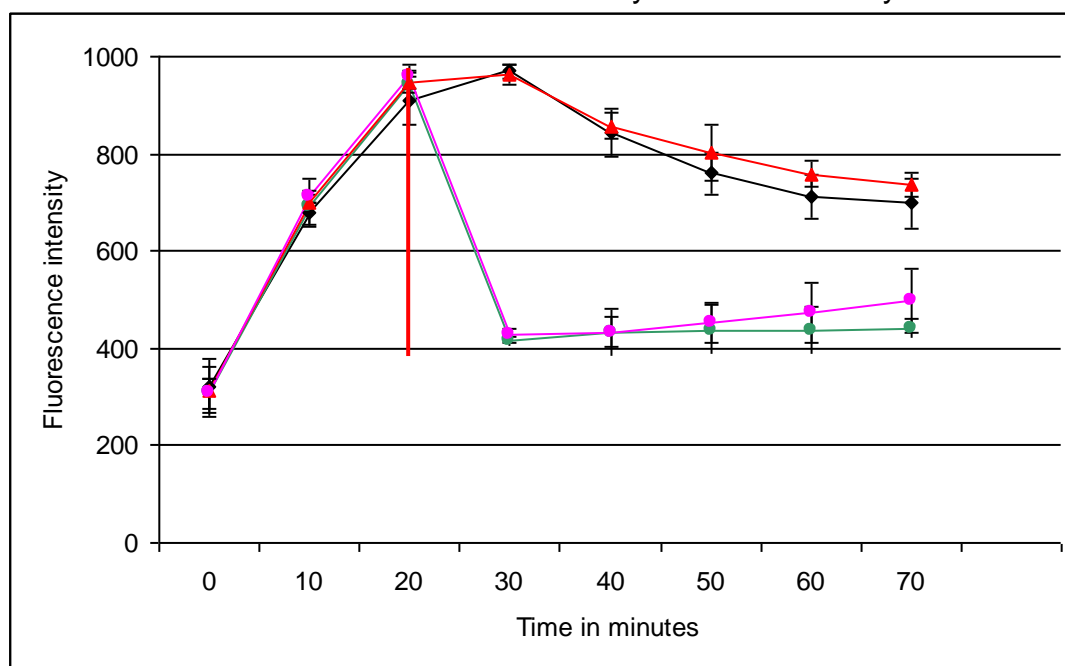


Figure 3.42. Uptake of EtBr by *L. monocytogenes* and efflux following addition of; ♦ = glucose, ▲ = glucose and reserpine, ■ = glucose and SAS, ● = glucose, SAS and reserpine as determined by fluorimeter. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm.

↓ = addition of glucose . n=3. SE shown.



Figure 3.42 shows no significant difference ( $p=0.7$ ) in the rate of efflux of EtBr following addition of glucose alone and glucose with reserpine. There is also no significant difference ( $p=0.9$ ) in efflux following addition of glucose in combination with SAS and glucose in combination with SAS and reserpine. Previous results suggested that reserpine was an inhibitor of efflux in *L. monocytogenes* (Figure 3.38c) but this was counteracted when the EPI was in combination with the detergent (Figure 3.39b) and the cells demonstrated an increase in resistance. It may be that the observations made here are due to an interaction between the glucose and reserpine that inhibits its activity, but this will need to be investigated further.

### **3.9. Composition of commercial detergents and carbon standards**

Commercially produced detergents, used in food industry cleaning procedures, are known to be mixtures of several different carbon chain lengths and it is hypothesised that it is the combination of carbon chain lengths that cause changes to occur in susceptibility of *E. coli* to BAC.

To investigate the composition of the detergents and the carbon chain standards, samples were analysed by mass spectrometry (MS).

During analysis by MS, some molecules break into fragments while others remain intact, depending on the functional groups and structure of the molecule. The detergent head groups of  $\text{NaSO}_4$  are lost through the weak bond to the carbon chain which undergoes further fragmentation and is represented by peaks showing molecular mass on a mass spectrum. Analysis of the molecular weights of fragments obtained can confirm lengths of carbon chains present.

The molecular weight of sodium n-decyl sulphate is  $260 \text{ g mol}^{-1}$ , which consists of a head group of  $\text{NaSO}_4$  at  $119 \text{ g mol}^{-1}$  and a carbon chain of  $141 \text{ g mol}^{-1}$ . Addition of fragment sizes  $83.1 + 56 = 139 \text{ g mol}^{-1}$  does not equal the molecular weight of the carbon chain exactly due to the loss of hydrogen ions but analysis of the mass spectrum (Figure 3.43a) shows a molecule of  $140.1 \text{ g mol}^{-1}$  which represents the intact tail of 10 carbons.

The molecular weight of sodium tetradecyl sulphate is  $316 \text{ g mol}^{-1}$ , which consists of a head group of  $\text{NaSO}_4$  at  $119 \text{ g mol}^{-1}$  and a carbon chain of  $197 \text{ g/mol}$ . Addition of other fragment sizes such as  $125.2 + 69.1 = 194.3 \text{ g mol}^{-1}$  do not equal the molecular weight of the carbon chain exactly, due to the loss of additional hydrogen ions. Although some fragment sizes added together are the same size as the carbon 12 tail, analysis of the mass spectrum (Figure 3.43b) shows molecules of  $196.1$  and  $197.2 \text{ g mol}^{-1}$ , which represent the intact tail of 14 carbons.

The molecular weight of sodium n-hexadecyl sulphate is  $344 \text{ g mol}^{-1}$ , which consists of a head group of  $\text{NaSO}_4$  of  $119 \text{ g mol}^{-1}$  and a carbon chain of  $225 \text{ g mol}^{-1}$ . Addition of other fragment sizes such as  $125 + 97 = 222$  and  $57 + 69 + 97 = 223 \text{ g mol}^{-1}$  do not add up to exactly the molecular weight of the carbon chain, due to the loss of additional hydrogen ions. Although some fragment sizes added together are the same size as the carbon 12 and carbon 14 tails, analysis of the mass spectrum (Figure 3.43c) shows molecules of  $224.1$  and  $225.3 \text{ g mol}^{-1}$  that represent the intact carbon 16 tail.

The molecular weight of sodium n - octadecyl sulphate is  $372 \text{ g mol}^{-1}$ , which consists of a head group of  $\text{NaSO}_4$  at  $119 \text{ g mol}^{-1}$  and a carbon chain of  $253$

g mol<sup>-1</sup>. Addition of other fragment sizes such as 167 + 83 = 250 and 139.1 + 111.2 = 250 g mol<sup>-1</sup> do not add up to exactly the molecular weight of the carbon chain, due to the loss of additional hydrogen ions. Although some fragment sizes added together are the same size as the carbon 12, 14 and 16 carbon tails, analysis of the mass spectrum (Figure 3.43d) shows molecules of 252.2 and 253.2 g mol<sup>-1</sup> that represent the intact carbon 18 tail.

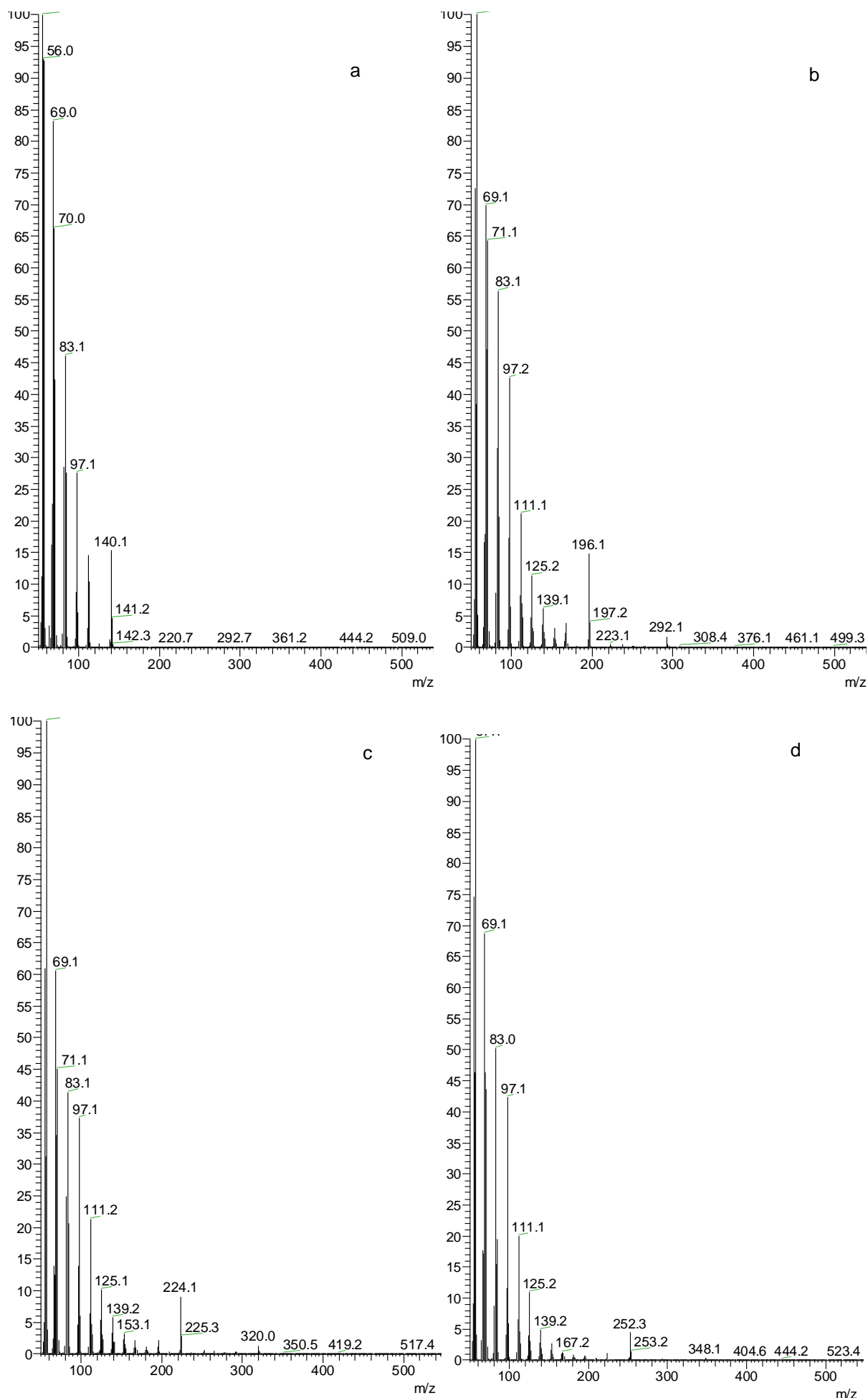


Figure 3.43. Mass spectrum of carbon chain standards; (a) sodium n-decyl sulphate, (b) sodium tetradecyl sulphate, (c) sodium n-hexadecyl sulphate, (d) sodium n-octadecyl sulphate.

The molecular weight of SDS is  $288 \text{ g mol}^{-1}$ , which consists of a head group of  $\text{NaSO}_4$  at  $119 \text{ g mol}^{-1}$  and a carbon chain of  $169 \text{ g mol}^{-1}$ . Addition of fragment sizes such as  $97.1 + 69.1 = 166.2 \text{ g mol}^{-1}$  and  $97.1 + 71.1 = 168.2 \text{ g mol}^{-1}$  do not equal the molecular weight of the carbon chain exactly, due to the loss of additional hydrogen ions but analysis of the mass spectrum (Figure 3.44a) shows molecules of  $168.1$  and  $169.3 \text{ g mol}^{-1}$  which represent the intact tail of 12 carbons.

The mass spectrum of SAS (Figure 3.44b) shows fragments of molecular weight  $168.0$ ,  $195.9$  and  $225 \text{ g mol}^{-1}$  that represent the intact tails of 12, 14 and 16 carbons that form the commercial detergent and the mass spectrum of SLES (Figure 3.44c) shows a fragment of molecular weight  $169.1 \text{ g mol}^{-1}$ , which represents the intact carbon 12 tails that form the commercial detergent.

FAE (figure 3.44d) and PEA (results not shown) are non-ionic detergents that have carbon tails of 12-16 carbons and varying numbers of ethylene oxide head units. It is therefore not possible to give a precise molecular weight or to determine from the mass spectrum the length of the carbon chain tail.

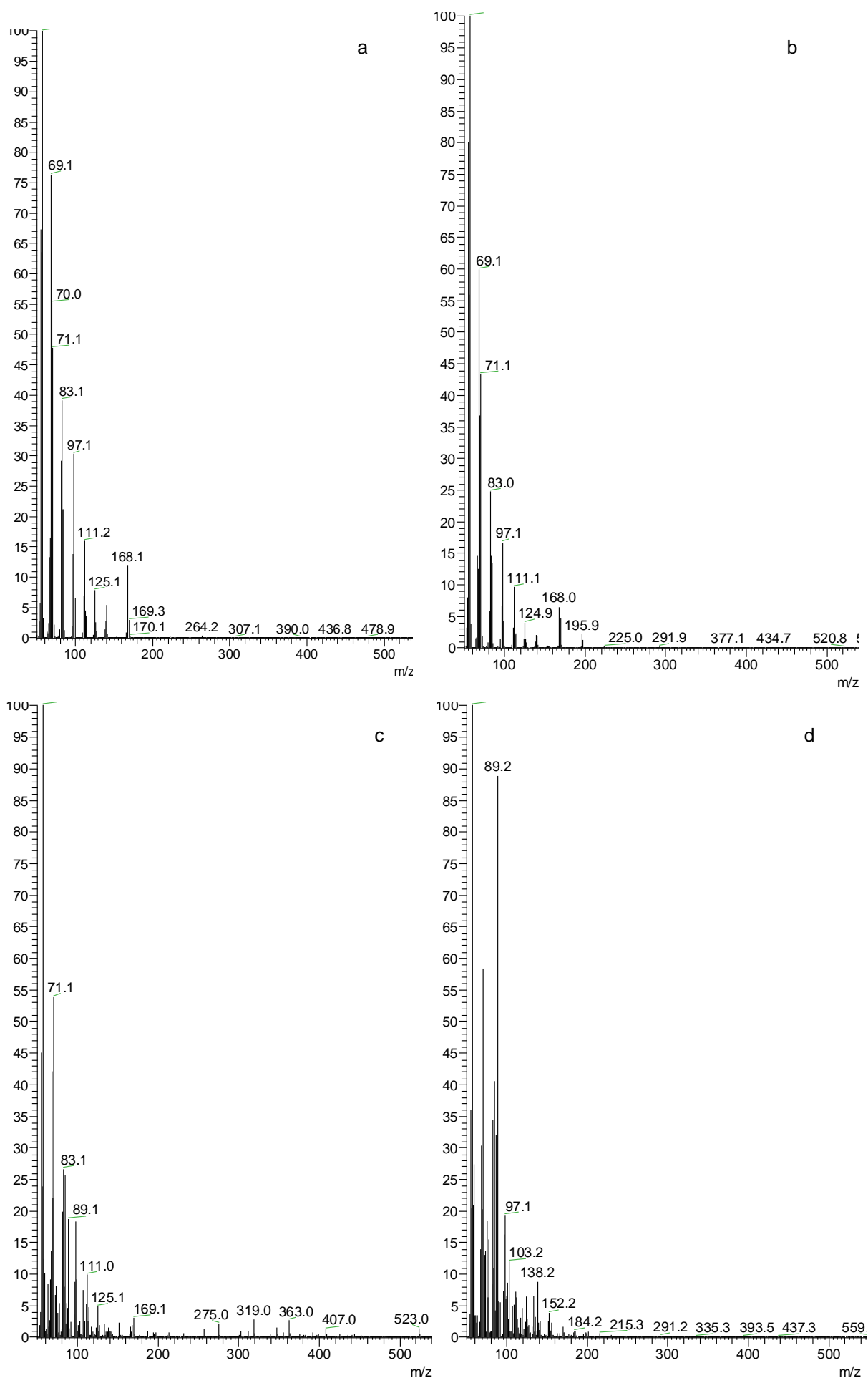
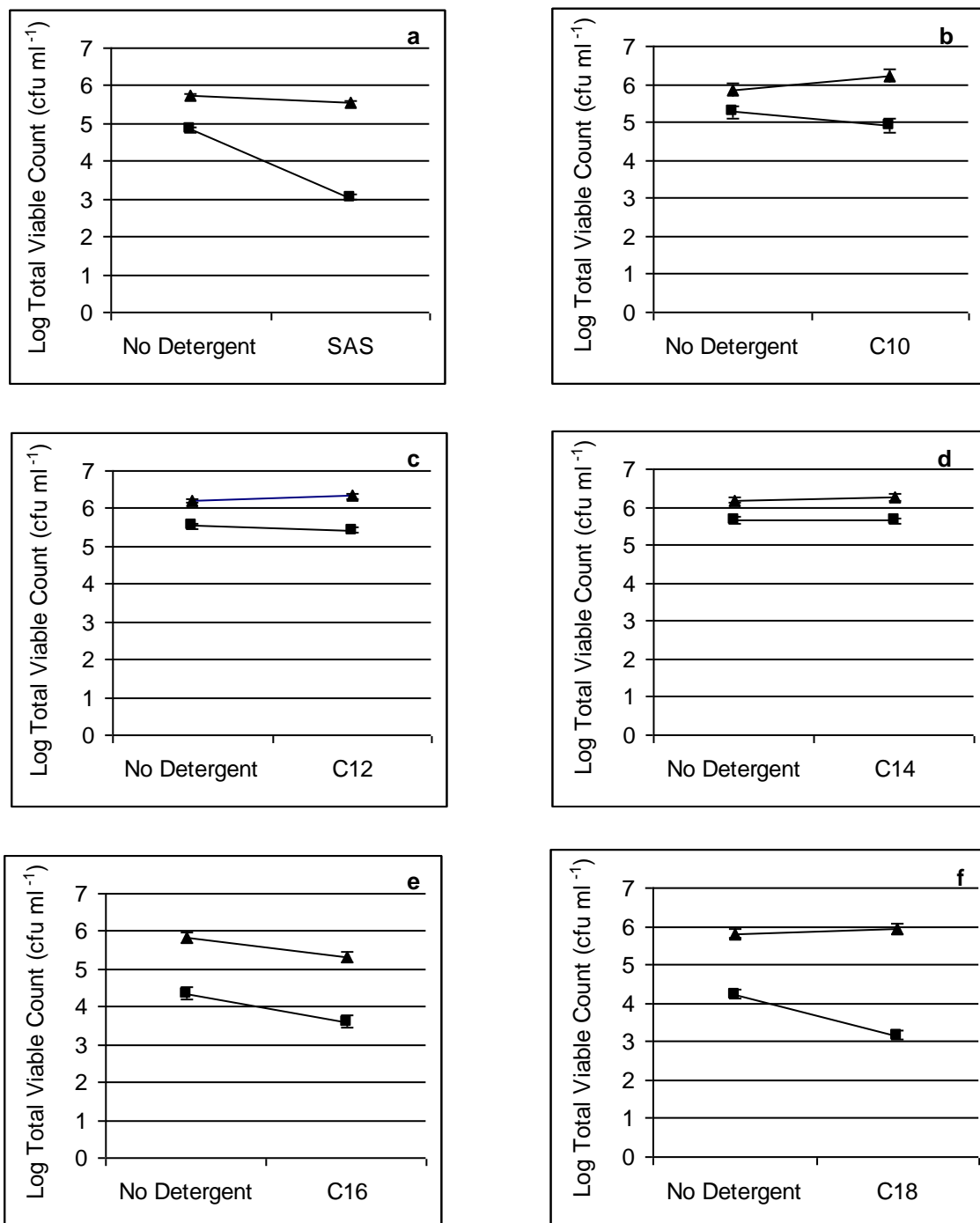


Figure 3.44. Mass spectrum of (a) SDS, (b) SAS, (c) SLES and (d) FAE.

### **3.9.1. Effect of SAS and carbon chain length standards on susceptibility of *E. coli* to BAC**

To further investigate why *E. coli* becomes more susceptible to disinfectant following treatment with anionic SAS and SLES and non-ionic FAE, the effect of different carbon chain length standards was investigated to determine whether the results observed could be attributed to one particular chain length. *E. coli* was treated with the homologous series of carbon chain length standards for 20 minutes followed by 5 minutes exposure to BAC.



Figures 3.45 a-f. Interaction plot of *E. coli* treated with SAS or carbon chain standards followed by BAC. ▲ = no disinfectant, ■ = disinfectant. n=9. SE shown.

In Figures 3.45 a-e it can be seen that only C16 had a significant effect on TVC of *E. coli* ( $p < 0.01$ ) while BAC significantly reduced TVC in all samples ( $p < 0.01$  for all). *E. coli* becomes significantly more susceptible to BAC



following treatment with SAS ( $p < 0.01$ ), which agreed with previous observations (Figure 3.1), and the only other interaction effect was seen with cells treated with C18 (Figure 3.43f) with a divergence of the lines showing that *E. coli* becomes significantly more susceptible to BAC ( $p < 0.01$ ).

### **3.9.2. Effect of SAS and carbon chain length standards on cell membrane permeability of *E. coli***

Investigations with the carbon chain lengths continued to determine whether particular carbon chain lengths of the detergents had an effect on cell membrane permeability that may affect susceptibility to BAC. *E. coli* was treated for 20 minutes with a homologous series of sodium sulphate standards with carbon chain lengths of 10, 14, 16 and 18 carbons, SDS which is a 12 carbon molecule and the detergents SAS and FAE. Excess detergent was removed from the cells by rinsing in Ringers solution. A change in permeability of the cell envelope was assessed with the fluorescent dye PI that was added to samples of the cells and fluorescence was observed by fluorimetric analysis.

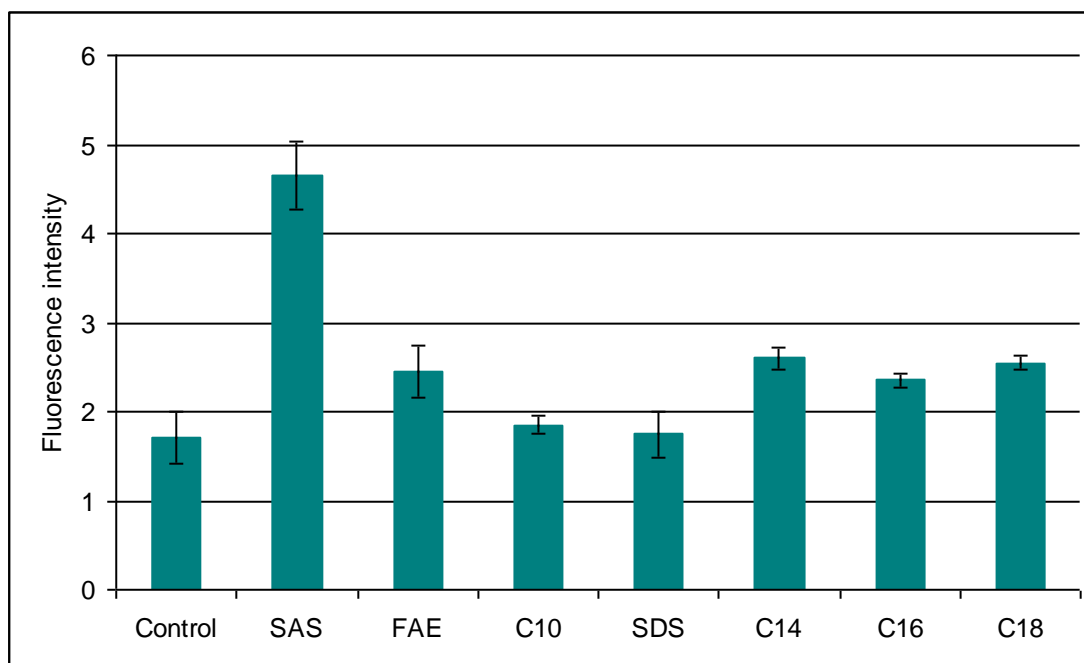


Figure 3.46. Effect of carbon chain standards on permeability of *E. coli* as determined by fluorimetric analysis. The fluorochrome propidium iodide was used with an excitation wavelength of 536 nm and an emission wavelength of 617 nm.  $n=9$ . SE shown.

Figure 3.46 shows that SAS, FAE and C14, 16 and 18 had significant effects on cell membrane permeability of *E. coli* ( $p<0.01$ ) and the increase observed with SAS was significantly greater ( $p<0.01$ ) than the others. No change was observed with C10 and SDS (C12) ( $p=0.1$  and  $0.4$  respectively).

The objective of this study was to establish whether specific combinations of detergent and disinfectant impacted upon the biocidal activity of the disinfectant against persistent strains of *Escherichia coli* and *Listeria monocytogenes* and why. This could impact on future cleaning and disinfectant regimes in the food industry and ultimately the safety of food.

#### **4.1 Susceptibility of *E. coli***

The viability of *E. coli* was not affected by exposure to any of the detergents at the in use concentration recommended by suppliers for cleaning procedures (Figures 3.1, 3.3, 3.5, 3.7 and 3.9). This would be expected as detergents are designed to remove organic soil, before the application of disinfectant, and are not intended to be antimicrobial agents (Gibson *et al.*, 1999) although, they may be antimicrobial if the concentration was sufficiently high enough. Glover (1999) observed that the biocidal efficiency of surfactants depended on the type of organism used, and that anionic surfactants such as SDS and non-ionic alcohol ethoxylate had little biocidal action on all the organisms tested while Flahaut *et al.* (1996) stated that extreme detergent resistance is a property of Gram-negative bacteria. The disinfectant BAC significantly reduced the TVC in all experiments as the concentration used was chosen to give a measurable kill.

Combined treatments of SAS or FAE followed by BAC (Figures 3.1 and 3.5), significantly reduced the TVC of *E. coli* greater than the sum of the individual

treatments demonstrating an increase in susceptibility to BAC following detergent treatment. No such change in susceptibility was observed following treatment with SDS, SLES or PEA Figures (3.3, 3.7 and 3.9) though it may be expected that SDS and SLES, that are also anionic detergents, would have the same effect as SAS. Kramer and Nickerson (1984) observed that 200 of 208 *Enterobacteriaceae* were able to grow in  $\geq 5\%$  SDS while some strains of *Salmonella* and *E. coli* were resistant to the detergent. They concluded that detergent resistance was a common property in enteric bacteria, which may explain why no change was observed following SDS, SLES and PEA or it may be the properties of the detergents that determines their effect on susceptibility of *E. coli* to BAC.

An explanation for an increase in susceptibility to BAC may be the structure of the detergent molecule, or the composition of the detergent. Although there are no clear results for FAE (Figure 3.44d), MS analysis shows that SAS (Figure 3.44b) is a combination of chain lengths of 12, 14 and 16 carbons and it is hypothesised that the combination of different carbon chain lengths are able to penetrate the cell envelope to affect susceptibility while SDS and SLES (Figures 3.44a and 3.44c) comprise only 12 carbon chains and have no effect.

The observed increase in susceptibility may also be due to a synergy between the detergent and disinfectant where their combined effect is greater than the sum of the individual effects. Lehmann (1988) stated that resistance is less likely when two antimicrobial agents are combined and Denyer and Maillard (2002) suggest that chemicals working in combination could overcome the impermeable barrier of the Gram-negative LPS. Moore and Payne (2004)

reported that resistance is well documented for organisms treated with biocides alone and BAC resistance is not common among food associated Gram-negative bacteria. However, this does not take into account that the organisms are pre treated with detergent during cleaning procedures, which may have an effect on susceptibility to disinfectant treatment.

Combined treatments of SAS, FAE or SLES followed by NaDCC (Figures 3.10, 3.12 and 3.14) showed a significant reduction in the TVC of *E. coli* that was greater than the sum of the individual treatments and again demonstrated an increase in susceptibility to the disinfectant following detergent treatment. Maillard (2002) explains that biocides interact with and oxidise targets such as the thiol groups of cysteine residues that are essential for enzyme activity, and Coates (1977) reported that hypochlorite interacts strongly with all types of organic material to cause serious loss of activity. Dukan *et al.* (1999) stated that hypochlorous acid can cause damage to DNA that is lethal and it is extremely potent as a bactericidal agent. The results demonstrate that whatever effect the detergents have on the cell envelope, it enhanced the oxidising action of NaDCC. Due to the overall effects seen with NaDCC no more experiments followed with this disinfectant but were performed with BAC due to the differences observed.

When time of exposure to SAS and SDS was increased, the previously observed effects were reversed with no effect on susceptibility observed following 2 hours treatment with SAS (Figure 3.17), while a significant increase in resistance to BAC following 2 hours treatment with SDS (Figure 3.18).

The timing of events varied for the different detergents and one hypothesis is that for SAS a long exposure time results in development of apparent resistance, while SDS takes a longer time to have an effect on the cell membrane leading to increased susceptibility. The observed resistance may be by efflux, membrane alterations induced by detergent interaction or by the response of stress proteins. Ruseka *et al*, (1982) proposed that increased resistance to BAC of Gram-negative bacteria was due to increased cell envelope lipid content. Similarly, Mechin *et al*. (1999) attributed an increase in resistance of *P. aeruginosa* to a QAC, during adaptation experiments, to alterations in fatty acid proportions, which returned to pre-adaptation proportions following sub culturing without the QAC. They concluded that the biocide causes changes to lipid A of the LPS and that changes in the fatty acids enhance resistance by making it difficult for QAC to pass through the OM (Chapman, 2003). This may explain what is occurring to cells exposed to SAS for 2 hours as these changes would most likely occur over a period of time.

For SDS the main target is the cell membrane (Singer and Tjeerdema, 1993) where it leads to membrane damage, alterations in carbon metabolism and ultimately damage to organelles by solubilisation of membrane proteins (Sirisattha *et al.*, 2004) but again these changes may be time dependent and the effects only seen after exposure of 2 hours. Membrane damage may also take longer due to the single carbon chain length of 12 carbons in SDS compared to the combination in SAS.

Because SAS and FAE are commercially used detergents, their positive effect on susceptibility of *E. coli* to BAC was further investigated by extending the

treatment time to 4 hours. There was an increase in susceptibility of *E. coli* to BAC following 20 minutes and 1 hour treatment with SAS but no change was observed following 2 and 4 hours (Figures 3.16 a-d).

The observations made at 4 hours were due to a reduction in TVC of susceptible cells with SAS alone that may be due to the alkyl chain of the detergent inserting into and disrupting the LPS of the OM and then the inner membrane. This may take time as the Gram-negative cell wall provides a protective barrier that needs to be compromised for damage to occur. Moore *et al.* (2006) observed leakage of K<sup>+</sup> through progressive membrane damage of *E. coli* when exposed to C10E6 and C12E6 alcohol ethoxylates that increased with contact time of up to 2 hours.

Following 2 hours treatment with SAS (Figure 3.16c), *E. coli* changed from being more susceptible to BAC to no change in susceptibility that indicated that alterations had occurred. *E. coli* are known to efflux antimicrobial agents (Russell *et al.*, 1986; Ma *et al.*, 1995; Russell and Chopra, 1996) and an efflux mechanism could have been induced over the extended time of detergent treatment. However, the results show no change in susceptibility to BAC, rather than an increase in resistance, which would have been expected if efflux was involved and it is hypothesised that membrane changes have occurred. This may be through alterations in lipid content of the OM, or over a period of time, the detergents inserting into the OM and cytoplasmic membrane to reduce fluidity of the membranes (Moore *et al.*, 2006) and prevent access of the disinfectant to the cell. The same hypothesis applies to *E. coli* treated with FAE for up to 4 hours (Figures 3.19 a-d) where no further interaction effect was observed following 20 minutes exposure to the

detergent, again suggesting that changes are occurring in the cell wall, over the extended time, to protect against the effect of the disinfectant.

When the concentration of SAS was increased (Figures 3.24 a-c), 0.2, 0.4 and 0.8 % (v/v) had no effect on TVC of *E. coli* but there was a significant increase in susceptibility to BAC that was not concentration dependent above 0.2 % over the range tested. When treated with 0.1 and 0.2 % (v/v) FAE (Figures 3.25 a-b), there was no effect on TVC but there was a significant increase in susceptibility to BAC that was not concentration dependent above 0.1 %. At 0.4% FAE (Figure 3.25c) TVC was significantly reduced which may be due to the higher concentration disrupting the membrane as low concentrations may protect against leakage by surfactant monomers inserting into the cytoplasmic membrane to reduce fluidity (Moore *et al.*, 2006). When treated with BAC a reduction in susceptibility was observed which did not calculate as significant due to the number of cells killed by detergent treatment alone.

Overall, a 4-fold increase of SAS and FAE had no further effect on susceptibility of *E. coli* to BAC, which demonstrated there would be no advantage in increasing detergent concentration in the food industry cleaning procedures to influence disinfectant susceptibility.

Further investigations were required to try and determine the underlying mechanisms contributing to changes in susceptibility. These required the use of cells in suspension and susceptibility tests were repeated with suspended cells. The results (Figures 3.26 a and b) showed the same trends as for attached cells, which enabled investigations to continue.



To determine whether changes in susceptibility were due to cell membrane permeability, *E. coli* were treated to the detergents for up to 2 hours (Figure 3.28), before addition of PI and observation of fluorescence. PI is a fluorescent dye used to determine changes in cell membrane permeability that may occur due to changes in membrane composition (Muller *et al.*, 2000). It is only able to enter the membranes of cells that have become permeable due to its double positive charge and probably due to its size of 668.4 g mol<sup>-1</sup> compared to 394.29 g mol<sup>-1</sup> of EtBr that can readily pass through intact membranes.

A significant increase in fluorescence, indicating a change in cell membrane permeability of *E. coli*, was observed following 20 minutes treatment with all of the detergents except anionic SDS and SLES. SDS and SLES also had no effect on susceptibility of *E. coli* to BAC, nor PEA, which caused only a slight increase in fluorescence, and the results suggest a link between cell membrane permeability and susceptibility in *E. coli*.

There is evidence in the literature to show that changes in cell membrane permeability caused by detergents may not lead to loss of viability (Helander and Mattila-Sandholm, 2000; Lukac, 2010) but, can render cells more susceptible to the actions of disinfectants. Changes in cell membrane permeability may be caused by the hydrophobic tail of the detergent interacting with membrane lipids leading to leakage of components through alterations to cytoplasmic membrane structure (Lukac *et al.*, 2010). Changes such as alterations in LPS interactions (Denyer and Maillard, 2002) that are important in maintaining membrane integrity and resistance to cationic biocides (Tattaswart *et al.*, 1999) or, in the cross-linking of the peptidoglycan

(McDonnell and Russell, 1999) that confers mechanical strength to the cell wall (Denyer and Maillard, 2002) would lead to an increase in susceptibility of the cell to disinfectant.

Observations by Helander and Mattila-Sandholm (2000) and Maillard (2002), who cited Ayres *et al.* (1999), suggested that some compounds such as citric acid, antibiotics and EDTA, behave like permeablising agents by interchelating in the OM to modify its integrity and release components from the cells. Hancock and Chapple (1999) wrote that disruption of the tightly packed LPS assembly mediates self-promoted uptake of agents across the OM. The cation binding sites of the LPS are essential for integrity and strength of the OM of Gram negative bacteria (Nikaido and Vaara, 1985; Vaara, 1992) and the tight interactions between membrane proteins and lipids of the phospholipid layer are essential in maintaining functions of the membrane such as diffusion, electrochemical reactions (Palsdottir and Hunte, 2004) and reduced biocide uptake which is evident where a high  $Mg^{2+}$  content produces the strong LPS-LPS links (Russell, 2001). Russell (1998) agrees that removal of  $Mg^{2+}$  may render cells susceptible to disinfectants while Glover *et al.* (1999) suggested that the action of surfactants might be similar to that of chaotropic anions that disrupt water structure and perturb the lipid bilayer by denaturing proteins.

It is possible that SAS and FAE behave like chelating agents by permeating the OM and binding the cations that maintain the structure of the LPS and, although not toxic to the organisms themselves, the detergents could be sensitizing them by weakening the bonds of the OM phospholipids.

This may allow for increased activity of hydrophobic agents such as QACs, that would otherwise be excluded entry to the cell (Vaara, 1992; Alakomi *et al.*, 2000) to penetrate the membrane with the hydrophobic tail to cause loss of permeability and a reduction in electrical potential (Lukac *et al.*, 2010). Glover *et al.* (1999) observed that detergents significantly increased cytoplasmic membrane fluidity of both Gram-positive *S. aureus* and Gram-negative *P. mirabilis* in the order of non-ionic > cationic > anionic. Their investigation used probes to determine the effect of detergents on cell membranes that showed increased fluidity in both the outer and cytoplasmic membranes of *P. mirabilis* caused by interference in the packing of the phospholipid hydrocarbons. They proposed that if a detergent interacts with the surface of the membrane, and not the whole lipid bilayer, the effect might not be biocidal. While it may be assumed that an increase in cytoplasmic membrane fluidity would lead to cell death, no relationship was observed between an increase in membrane fluidity and biocidal activity of the detergents, which was dependent on the organism tested (Glover *et al.*, 1999).

Brown and Richards (1964) observed enhanced antibacterial activity of BAC on *P. aeruginosa* following treatment with non-ionic Polysorbate (Tween) 80 while the detergent alone had no effect on cell viability. They suggested the detergent was causing changes to occur in the organisation of the cell membrane / envelope and through further experimentation observed that non-ionic agents reduced electrostatic resistance and increased cation permeability. Brown and Winsley (1971) later suggested that non-ionic detergent caused alterations to the OM lipids of *P. aeruginosa* that allowed

easier access of cationic polymyxin to the inner membrane. These results were supported by Hugo *et al.* (2004) who reported that anionic surfactants induce changes in the permeability of the OM leading to increased sensitivity of some organisms to antimicrobial agents. In addition Moore *et al.* (2006) stated that non-ionic detergents could increase permeability of the cell membrane causing leakage of cellular components with the head group of ethylene oxide units reacting readily with functional groups on proteins to cause irreversible changes in protein structure (McDonnell and Russell, 1999).

*E.coli* are known to demonstrate efflux activity via a tripartite system that spans the cell wall (Figure 1.5) and it would be expected that following entry through the OM, the disinfectant would pass through the periplasm and be captured by the transporter protein of the efflux pump that is sited in the inner CM (Li and Nikaido, 2009). However, an increase permeability of the OM, due to the effect of the detergent (Figure 3.28), may lead to a spontaneous and rapid influx of disinfectant that overwhelms the capability of the efflux system that would normally aid in resistance. An accumulation of disinfectant in the periplasm would then be able to pass through the CM to cause cell death and demonstrate an increase in sensitivity.

The initial increased permeability of cells treated with SAS and FAE was shown by experiments using PI experiments to decrease after 2 hours exposure (Figure 3.28) suggesting that bonding initially disrupted by the detergents is reorganised or, it may be due to a response by stress proteins over the extended period of exposure to the detergent.

Palsdottir and Hunte (2004) reported that when the structure of the membrane is rearranged, the cell membrane is kept sealed by interactions between the membrane proteins and the lipid bilayer. Nixdorff *et al.* (1978) treated LPS with an ionic detergent, at a concentration that would be expected to disrupt the plasma membrane, and observed aggregation of LPS that they suggested offered protection against the action of surfactants. Again the results suggest a link as on initial exposure to SAS and FAE, there is an increase in cell membrane permeability that increases susceptibility to BAC but following 2 hours, a reduction in permeability is observed that correlates with a decrease in susceptibility to BAC (Figures 3.18 and 3.19).

The link between increase/decrease in susceptibility and increase / decrease in permeability, as a result of detergent treatment, agrees with the observations of Ishikawa *et al.* (2002) and their work with *E. coli* and, with Mechin *et al.* (1999) and their work with *Pseudomonas aeruginosa*. In contrast, Langsrud *et al.* (2003) studied membrane permeability in Gram-negative bacteria adapted to BAC and observed no correlation between a decrease in membrane permeability and resistance.

Cell membrane permeability increased following 2 hours treatment with anionic SLES (Figure 3.28), which cannot be linked to changes in susceptibility. The increase in permeability may be due to the detergent inserting into and interfering with the structure of the LPS although the same results as SDS could have been expected as they both have chains of 12 carbons.

Although cells treated with SDS showed an increase in resistance to BAC following 2 hours treatment (Figure 3.17) no change in cell membrane

permeability was observed (Figure 3.28), which may be due to SDS targeting the cell surface through adsorption rather than intracellularly (Tattaswart *et al.*, 1999) to interfere with BAC interaction.

When the concentration of SAS and FAE was increased (Figure 3.30a and 3.31a), there was a significant increase in cell membrane permeability by approximately 2-fold compared to the control cells but no difference in effect between each of the concentrations. This correlates with results in Figure 3.24 where none of the concentrations had a greater effect on susceptibility than another over the range tested and shows that up to a 4-fold increase in concentration of the detergents, above the in use concentration has no added effect. Again the results suggest it would be of no benefit to increase the concentration of detergent used in cleaning procedures in the food industry.

Cell surface hydrophobicity (CSH) was investigated as changes occurring at the cell surface can influence the approach of a molecule to the cell and its interaction at the surface. CSH is important in adhesion of organisms to surfaces (Li and McLandsborough, 1999; Brown, 2005) and interactions that are integral in the organisation and integrity of the membrane components. Poole (2002) suggested that changes in the cell membrane permeability and CSH influence disinfectant resistance and Kim *et al.* (2007) reported that changes in CSH can affect permeability of the cell membrane, which can lead to cell death. Li and McLandsborough (1999) stated that the hydrophobicity of bacteria can change with variation in composition of suspension media and the results of the hydrophobicity studies show (Figure 3.32a) that the detergents appear to bring about gross changes to the surface of *E.coli* that causes them to become significantly more hydrophilic except for cells treated

with FAE, which remained unchanged. The Gram-negative OM is highly hydrophobic and provides a permeability barrier to hydrophilic agents (Stavri *et al.*, 2007). CSH decreased in *E. coli* following treatment with SAS and SDS, which for SAS suggests a link to the increase in cell membrane permeability, through interaction of the carbon chains with cell envelope, and susceptibility to BAC. For SDS, where no increase in cell membrane permeability was observed, the results support the earlier suggestion that the changes are due to the detergent molecules adsorbing to the surface of the cell to bring about changes in cell surface properties and hydrophobicity (Tattaswart *et al.*, 1999). Skvarla *et al.* (2002) stated that cationic surfactants adsorb to the negatively charged surface of the cells by the cationic head groups, which increases cell surface hydrophobicity due to the hydrocarbon tails being orientated toward the aqueous environment. This may provide information about the orientation of the detergents as if the anionic detergents were adsorbing to or incorporating into the cell surface by their non-polar moieties, the negatively charged head groups would be orientated towards the aqueous medium increasing the overall negative surface charge and reversing the hydrophobic character of the cell wall to hydrophilic (Skvarla *et al.*, 2002). This may then influence the cells response to BAC (Sikkema *et al.*, 1995) as the cationic disinfectant is likely to be 'mopped up' by the increased negative charge on the cell or, prevented from inserting into the lipid membrane to cause damage (Marcotte *et al.*, 2005). No change in CSH was observed for *E. coli* treated with FAE, which is probably due to the lack of charge on the detergent molecule. Bond *et al.* (2005) studied interactions between membrane proteins and detergents and observed that detergent

adsorbed to the OM protein Omp A and sequestered the non-polar regions of protein away from the water suggesting that the change in CSH is caused by changes in hydrophobic interactions that are important in protein structure. Gardner and Peel (2001) observed that chlorhexidine strongly adsorbed to the surface of *E. coli* and reduced the negative charge on the cells that resulted in changes in cell structure and metabolism. As the chlorhexidine penetrated the cells leakage ceased as precipitation occurred within the cytoplasm. Similarly, McDonnell and Russell (1999) reported that a range of compounds including QACs have been shown to cause cytoplasmic protein coagulation.

Modifications in the thickness and the amount of cross-linking of peptidoglycan (McDonnell and Russell, 1999), alterations in fatty acids and changes in cell surface charge (To *et al.*, 2002), that may be responsible for the change in CSH have been seen to prevent the entry of QACs in Gram-negative organisms. It is likely that there will be more than one factor involved in the changes that have been observed.

It was previously hypothesised that the combination of chain lengths was responsible for changes seen in susceptibility and to determine this *E. coli* was treated with sodium alkyl sulphate standards with carbon chain lengths of 10, 14, 16 and 18, and SAS, followed by BAC (Figures 3.45a-f). A significant increase in susceptibility was observed following treatment with SAS (Figure 3.45a), which agreed with previous results and demonstrated that the formulation of the detergent was not only effective as a detergent, but also has antimicrobial effects. An increase in susceptibility was also observed following treatment with C18 (Figure 3.45f), but no changes were observed with any of the other carbon chain lengths. *E. coli* was also treated with



sodium alkyl sulphate standards with carbon chain lengths of 10, 14, 16 and 18 and the detergents SAS, SDS (C12) and FAE, followed by BAC to determine the effect of chain lengths on permeability Figure 3.46). SAS, FAE and C14, 16 and 18 caused an increase in cell membrane permeability but no change was seen with SDS and C10.

Overall, increases in susceptibility and permeability were caused by C18 and SAS which suggests the longer chain length and the combination of chain lengths are the most effective. As SAS is a mixture of carbon chain lengths, it may be the combination of these that is leading to the observed changes in susceptibility, through cell membrane permeability. It is possible that the range of different lengths are able to permeate the LPS of the cell wall more effectively than a single chain length to cause more disruption. Although, changes were also observed with C18 this is probably due to its longer length disrupting the cell membrane to allow access of the hydrophobic disinfectant. Properties of alkyl sulphates vary according to chain length and longer chain lengths of the detergents may penetrate further into the LPS to bind with the cations that maintain the integrity of the LPS. When referring to the antimicrobial action of QACs, Lukac *et al.* (2010) reported that activity increased with chain length. Chain length may also account for the increase in permeability by FAE, which is intrinsically a longer molecule due to the ethylene oxide units of the non-ionic head group. According to Moore *et al.* (2006), non-ionic detergents are known to cause changes to cell membrane permeability that leads to leakage of cellular components such as  $K^+$ . They investigated the effect of chain length of detergent on the cell membrane by treating *E. coli* and *S. aureus* to a homologous series of alcohol ethoxylates

(non-ionic surfactant) with tail groups of 10-16 carbons and observed that the bacteriostatic activity of the alcohol ethoxylates was greater against the Gram-positive than the Gram-negative organism. In this study the detergents were used at a concentration of 0.6 mM and at 0.2-0.5 mM Moore *et al.* (2006) observed a greater increase in cell membrane permeability and leakage of cytoplasmic constituents caused by the 10 and 12 carbons than the 14 and 16 carbon lengths. This was attributed to the shorter carbon tails being relatively less hydrophobic, which may explain change of CSH to hydrophilic, and able to cross the outer membrane via porins. Longer carbon tails, which were more lipophilic, would have less probability of passing through the aqueous porin channels. The leakage was however reduced at lower concentrations, which was attributed to detergent monomers inserting into the cytoplasmic membrane to reduce membrane fluidity, while membrane disruption was increased at higher concentrations. They also observed continuing membrane damage of *E. coli* with increased contact time to the 10 and 12 carbon chain alcohol ethoxylates, which would correlate with the increase in susceptibility to BAC following 2 hours treatment with SDS. Kabara (1978) reported that the antimicrobial activity of lipophilic groups is dependent on optimum chain length, which varies according to the organism, with Gram-negative organisms affected by the lower chain lengths while the longer chain lengths affect Gram-positive organisms.

#### **4.1.1 *E. coli* conclusion**

SAS and FAE cause *E. coli* to become more susceptible to BAC, which appears to be linked to increases cell membrane permeability caused by the

detergents. Results suggest that the increase in permeability is caused by the combination of carbon chain lengths in SAS and the longer molecule of FAE that permeate the cell envelope of *E. coli* to render the organism susceptible to the action of the disinfectant. SDS being chains of 12 carbons targets the surface of the cell rather than disruption of the membrane. Although the change in CSH does not appear to have any influence on susceptibility of the cells to disinfectant, there is evidence that the detergent is influencing overall cell surface properties.

The oxidizing action of NaDCC is enhanced by the pre treatment of cells with all of the detergents.

#### **4.2 Susceptibility of *L. monocytogenes***

A significant reduction in the TVC of *L. monocytogenes* was observed after treatment with the anionic detergents SAS, SDS and SLES (Figures 3.2, 3.4 and 3.8), but FAE (Figure 3.6) had no effect on viability. This was not observed with *E. coli* and is most likely to be due to the structural differences in the cell walls.

Surfactants are not generally considered to have antibacterial action however, where they have been recognised as having antimicrobial effects, it has been greater against Gram-positive bacteria than Gram-negative bacteria (Galbraith *et al.*, 1971; Glover *et al.*, 1999; Moore and Payne, 2004). Glover *et al.* (1999) investigated the effect of surfactant action on the membranes of *P. mirabilis* and *S. aureus* and observed the highest biocidal effect with the non-ionic surfactant on *S. aureus*. This does not agree with our observations for non-ionic FAE and *L. monocytogenes* but they concluded that the biocidal

efficiency of detergents was organism dependent. *L. monocytogenes* that survived treatment with the anionic detergents were observed to be significantly less sensitive to subsequent treatment with BAC. QACs can be inactivated by the presence of anionic detergents (Lehmann, 1988) and Heinzl (1988) stated that chemical interactions of antimicrobial agents with other compounds might result in a decrease of efficacy and simulate a resistance of organisms. This suggests that if the detergent was attaching to the surface of the cell, it may mop up and inactivate the disinfectant to give a perceived resistance. Bessems (1998) also demonstrated that the efficacy of disinfectants was dependent on the interaction of specific disinfectants with test microorganisms and practical conditions such as water hardness, time and temperature.

However, the reduction in TVC following the combined treatment was significantly less than the sum of the individual treatments suggesting a marked increase in resistance in detergent treated cells. According to Mereghetti *et al.* (2000), tolerance in *L. monocytogenes* may be the result of modifications of the surface of the cell and McDonnell and Russell (1999) speculated that this tolerance was due to changes occurring in the peptidoglycan in the cell wall. Gram-positive bacteria can also produce extracellular lipoteichoic acids which, being lipophilic, may interact with detergents or prevent penetration of sanitizers (Hammond *et al.*, 1984 cited by Frank and Koffi, 1990), although it is not confirmed if these changes can occur in the short time of exposure to the detergent.

It is hypothesised that the anionic detergents may be inducing an efflux mechanism in *L. monocytogenes* that significantly reduces intracellular BAC

concentration and hence apparent susceptibility as a rapid response after only 20 minutes treatment with detergent. Flahaut *et al.* (1996) reported a significant increase in resistance of *Enterococcus faecalis* to SDS following 5 seconds exposure and determined that the tolerance was protein synthesis independent while Begley *et al.* (2002) observed adaptation of *L. monocytogenes* to bile acids and SDS after 5 seconds treatment and identified the genetic locus involved in the response. Such studies support the hypothesis of a rapid detergent induced response such as efflux that protects the cell against subsequent exposure to antimicrobials.

No change in susceptibility was observed following treatment with FAE (Figure 3.6), which differs to the observations made with *E. coli* and shows that the non-ionic detergent is interacting differently with the cell envelopes of the two organisms. The observed changes may be attributed to the charge on the detergent molecule but factors such as length of the hydrophobic chain of the detergent and hydrophobicity of the cell should also be considered.

Combined treatments of SAS, FAE or SLES followed by NaDCC (Figures 3.11, 3.13 and 3.15) showed significant reductions in the TVC of *L. monocytogenes* that was greater than the sum of the individual treatments and again demonstrated an increase in susceptibility to the disinfectant following detergent treatment. The results agreed with *E. coli* demonstrating that the oxidising action of NaDCC was independent of detergent type and had the same effect despite differences in cell wall structure of the organisms. Due to their commercial use, increased time of exposure of SAS (Figures 3.20 and 3.21) and FAE (Figures 3.22 and 3.23) on susceptibility of

*L. monocytogenes* was assessed. A significant reduction in TVC, and susceptibility of the organisms to BAC, was observed at 20 minutes and 2 hours exposure with SAS and no difference was observed between the times again suggesting that a rapid response from *L. monocytogenes* led to the reduction in susceptibility. Although the reduction in susceptibility occurred very quickly, Aase *et al.* (2000) observed immediate efflux of EtBr from *L. monocytogenes*, on the addition of glucose, when studying mechanisms of resistance.

The significant reduction in TVC following 2 hours exposure with FAE (Figure 3.23) agreed with observations by Moore *et al.* (2006) that non-ionic detergents were increasingly bacteriostatic against Gram-positive compared to Gram-negative over time. No change in susceptibility was observed at either time, which differed to cells treated with SAS, and suggests that the charge on the anionic detergent molecule is an important influence in the induction of an efflux mechanism.

Investigations continued to try and determine the mechanisms that were causing changes to occur in susceptibility of *L. monocytogenes* to BAC by first looking at the effects of increased time of exposure to detergent on cell membrane permeability.

A significant increase in permeability, as shown by an increase in fluorescence, was observed in cells treated with SAS compared to control cells, but there was no difference in effect between the times (Figure 3.29). The fluorescence emitted was significantly lower than that seen with *E. coli* that may be due to a lipopolysaccharide like substance that Wexler and

Oppenheim (1979) isolated from the surface of *L. monocytogenes* that they hypothesised may decrease the permeability of the cell envelope.

The reduction in susceptibility may be due to the efflux system of Gram-positive-organisms, which is a single component that spans the CM, compared to the tripartite system of *E. coli*. While the disinfectant would have to cross the OM and periplasm of *E. coli* to be captured by the transporter protein, efflux by *L. monocytogenes* may be induced by the detergent binding to the cavity of the pump in the membrane bound proteins (Eswaran *et al.*, 2004), to activate efflux (Borges-Walmsley and Walmsley, 2001) before exposure to disinfectant occurs. If the pump is activated prior to disinfectant treatment this may lead to the observed increase in resistance. The binding of the detergent and / or the effect of efflux may explain the relatively small increase in permeability observed in *L. monocytogenes*.

The significant increase in cell membrane permeability following treatment with FAE (Figure 3.29) increased up to 2 hours but no change in susceptibility to BAC had been observed over the same times (Figures 3.22 and 3.23). The results differ to those seen with SAS and support the hypothesis that it is the charge on the anionic molecule that is important in the induction of an efflux mechanism. During efflux experiments potassium chloride was added as a stimulatory cation to induce efflux of EtBr (Jones and Midgley, 1985) and Cairney and Smith (1993) included K<sup>+</sup> to stimulate phosphate efflux when studying the influence of monovalent cations on efflux.

When investigations were performed into the effects of increased concentration of detergent on cell membrane permeability (Figures 3.30b and 3.31b), a significant increase was observed with SAS and FAE but there was

no difference between the concentrations used and no increase above the in use concentrations of 0.2 and 0.1 % respectively. The results correlate with previous observations where FAE causes a greater increase in cell membrane permeability than SAS, but has no effect on susceptibility, and supports the hypothesis that it is the charge on the SAS that influences induction of efflux and not the extent of cell membrane permeability.

As no change in permeability had been observed with a change in concentration, no experiments were carried out to compare increase in concentration on susceptibility of *L. monocytogenes*.

Overall the results show that up to a 4-fold increase in concentration of the detergents, above the in use concentration, did not have an increased effect on membrane permeability and there was no significant difference in permeability observed between the concentrations. Again the results suggest it would be of no benefit to increase the concentration of detergent used in cleaning procedures in the food industry.

The results of the hydrophobicity studies show that for *L. monocytogenes* (Figure 3.32 b), all of the detergents bring about gross changes to the surface of the cells that causes them to become significantly more hydrophilic. To *et al.* (2002) observed changes in cell surface properties of parent and adapted strains of *L. monocytogenes* to BAC and their study found that both strains were hydrophilic and suggested that changes in cell surface properties may act to repel BAC away from the cell. This could be a contributory factor in the reduction in susceptibility of cells treated with the anionic detergents. However, it is thought that another mechanism such as efflux may be present



as Jarlier and Nikaido (1994) suggested that the cell wall barrier alone would not be able to provide significant levels of antimicrobial resistance.

Braoudaki and Hilton (2005) observed that a reduced susceptibility associated with changes in CSH of *Salmonella* adapted to erythromycin, BAC and triclosan, was strain specific. They also concluded that CSH and active efflux could contribute to resistance of *S. enterica* to the antibacterial agents studied.

It was hypothesised that the observed decrease in susceptibility of *L. monocytogenes* to BAC was due to an efflux mechanism as the TVC of the combined treatments was greater than the sum of the individual treatments suggesting some mechanisms of resistance. Efflux is an important intrinsic mechanism in resistance (Schweizer, 2003) and efflux pumps are encoded chromosomally (Stavri *et al.*, 2007) or are acquired due to antimicrobial pressure (Marquez, 2005). Soumet *et al.* (2005) observed resistance to QACs in 42 % of *L. monocytogenes* strains tested by MIC assessment and their work on EtBr accumulation assays found that strains resistant to BAC and EtBr demonstrated efflux. Although several multi drug efflux pumps have been characterized, they speculated it would be difficult to determine which type of pump is active. Paulsen *et al.* (2001) and Aase *et al.* (2000) also reported that BAC resistance was mediated by a proton motive force efflux pump and Mata *et al.* (2000) provides evidence of the *mdrL* gene encoding an MDR efflux pump that is able to expel antibiotics and EtBr from *L. monocytogenes*. Resistance to QACs via MDR pumps was observed with *Staphylococcus* spp (Heir *et al.*, 1999) and *L. monocytogenes* (Aase *et al.*, 2000). In this study investigations were carried out to try and determine

whether the observed resistance to BAC by *L. monocytogenes* was due to an efflux pump induced by and responding to the detergent, as has been reported for disinfectants.

Initial efflux experiments on *L. monocytogenes* (Figure 3.33) established that efflux of EtBr was achieved through the addition of glucose, which was confirmed when testing the effect of temperature on efflux (Figure 3.34 a and b). When efflux was initiated with glucose plus detergents (Figure 3.35), there was a more rapid efflux of EtBr from cells with SAS added, suggesting that the detergent promoted efflux while cells with FAE added continued to uptake EtBr.

To continue investigations, cells were pre-treated with detergent prior to loading with EtBr (Figure 3.36) and there was a significantly greater uptake by cells pre-treated with SAS compared to FAE. It may have been expected from previous observations that SAS would induce efflux to prevent uptake of EtBr but the cells were depleted of energy during preparation for the experiment and uptake was not affected. The greater uptake by SAS treated cells does not link with cell membrane permeability where FAE causes a greater increase. Alternatively it is hypothesized that the greater uptake is related to the change in CSH but EtBr is a hydrophobic compound and there would be a natural repulsion between EtBr and the hydrophilic cell surface. However, Bhattachatya and Mandal (1997) investigated the effect of surfactants on the DNA binding of EtBr and observed that cationic surfactants destabilised the EtBr-DNA complex but in the presence of the anionic surfactants the complex remained stable. It may be that pre treatment of *L. monocytogenes* with the detergents affected the binding of EtBr to DNA with FAE inhibiting the EtBr-

DNA complex while it remained stable with SAS and more fluorescence was emitted. This is also supported by the results in figure 3.35 where there is continued increase in fluorescence from untreated cells to which glucose and FAE were added. This experiment had been carried out many times and the same observations made yet, when the cells were pre treated with the FAE, no increase in fluorescence was observed indicating no further binding of EtBr to DNA.

The increase in the rate of efflux from cells to which SAS was added supports the hypothesis of detergent induced efflux of disinfectant as, while some efflux pumps are expressed constitutively, others are induced in response to a substrate (Levy, 2002; Stavri *et al.*, 2007). Thanabalu *et al.* (1998) reported assembly of the TolC-HlyD pump in *E. coli* being induced by bacterial endotoxins and Stavri *et al.* (2007) reported that the MexXY-OprM pump of *P. aeruginosa* is induced in the presence of any of its substrates. It is possible therefore that the detergents could be substrates for an efflux pump in *L. monocytogenes* as Piddock (2006b) stated that bile salts induced expression of efflux pumps in enteric bacteria and Taylor *et al.* (2012) wrote that bile salts were a substrate of the RND efflux pumps in *Vibrio cholerae*. Several other studies suggested that bile salts and their components induced and upregulated expression of RND efflux systems (Chatterjee *et al.*, 2004; Bina *et al.*, 2008; Cerda-Maira *et al.*, 2008) and Rouquette *et al.* (1999) observed that Triton X up-regulated expression of the RND pump in *Neisseria gonorrhoeae*.

Paulson (2003) described MDR efflux transporters as having a large hydrophobic cavity able to accommodate hydrophobic substrates of different

structures but identification of the amino acid residues is still needed to understand the interaction with the substrate, and between intermolecular and intermolecular conformational changes (Nikaido and Pages, 2012).

Further experiments compared the effect on susceptibility of SAS and the EPIs, CPZ and reserpine (Figures 3.38 a-c). *L. monocytogenes* becomes significantly less susceptible to BAC following treatment with SAS but significantly more susceptible following treatment with CPZ and reserpine suggesting that *L. monocytogenes* may have an efflux system, inhibited by these EPIs, that pumps out BAC. The effect of the EPIs to make the cells more sensitive to BAC indicates the role of efflux in susceptibility and it is suggested that SAS may activate efflux resulting in the observed reduced sensitivity. This was followed by treating *L. monocytogenes* with SAS in combination with CPZ or reserpine (Figures 3.39 a and b). While an increase in sensitivity was expected, the same decrease in susceptibility was observed with both combinations as with SAS alone demonstrating that the EPIs did not inhibit efflux in the presence of the detergent. Possible explanations for this could be reduced bioavailability of the EPIs through the detergent either binding to the surface of the cell to prevent access or, interacting directly with the EPI. Alternatively, the detergent may bind to the efflux pump to impair the effectiveness of the EPIs that block the pump by binding to it, inhibit ATP or disrupt proton motive force (Marquez, 2005). CPZ inhibits the binding of calcium to proteins that are essential in ATPase activity and energy production for efflux as shown by Martins *et al.* (2011) who observed that while CPZ resulted in accumulation of EtBr in *E.coli*, this was prevented by the addition of  $\text{Ca}^{2+}$ . Addition of EDTA that binds divalent cations also

resulted in accumulation of EtBr and confirmed the presence of calcium efflux. If the anionic detergents bind to the EPIs, this may prevent their inhibition of calcium binding to proteins.

Reserpine has a molecular weight of  $608.68 \text{ g mol}^{-1}$  and CPZ of  $318.86 \text{ g mol}^{-1}$  and both structures contain benzyl or triazine rings. Compared to SDS for example, which has a molecular weight of  $288.37 \text{ g mol}^{-1}$  and a long chain carbon tail, the EPIs are large, heavy molecules and it may be that the detergent has easier access to the pump where it acts as a substrate to induce efflux that cannot then be inhibited by the EPI. The size of the molecules may also determine the effects of EPIs on cell membrane permeability as CPZ had no effect on cell membrane permeability on its own and no added effect when in combination with SAS (Figure 3.40). The effect of reserpine on cell membrane permeability was not investigated.

When CPZ was added to loaded cells in combination with glucose, no efflux of EtBr was observed suggesting that the EPI had inhibited the efflux mechanism (Figure 3.41). However, when CPZ was added in combination with SAS and glucose, efflux was activated to the same extent as SAS alone, supporting the susceptibility data that CPZ is not effective in the presence of SAS.

No inhibition of efflux was observed when reserpine was added in combination with glucose, as efflux was the same as when glucose was added alone (Figure 3.42). When reserpine was added in combination with SAS and glucose, the same rate of efflux was observed as with SAS alone and from these results it appears that reserpine is not an inhibitor of an efflux mechanism in

*L. monocytogenes*. This contradicts earlier results and those of Romonova *et al.* (2006) who observed a decrease in the MIC for BAC, of *L. monocytogenes* incubated with reserpine, which inhibits efflux pumps in the RND family, major facilitator family and the ATP binding cassette. However, although Lomovskaya *et al.* (2001) observed inhibition of MDRs by reserpine in Gram-positive bacteria, Schmitz *et al.* (1998) reported it had no effect on 50% of the *L. monocytogenes* strains tested which was attributed to reserpine resistance preventing the EPI from blocking the efflux mechanism. Soumet *et al.* (2005) investigated BAC resistant strains of *L. monocytogenes* in the presence of reserpine and their results suggested that other mechanisms such as changes in the outer membrane might contribute to resistance. The observation that reserpine was active when added alone, but was ineffective when added in combination with glucose, suggests that the combination may have affected its inhibitory action. Sonnet *et al.* (2012) investigated EPIs known to block RND efflux of fluoroquinolone in *P. aeruginosa* and observed that a reduction in MIC<sub>50</sub> by the EPI was dependent not only on the organism but also the combination of EPI and agent it was associated with, which could bind to different sites of efflux pumps. Bohnert *et al.* (2010) demonstrated competition between fluoroquinolone and EPIs for efflux pumps yet Martins *et al.* (2011) observed that glucose added with CPZ did not obviate the effect of CPZ on efflux. It may be that the observations made in this study are due to the differing structures of reserpine and CPZ and whether or not they form a complex with glucose that is unable to bind and influence efflux.

They concluded that it would be doubtful that an EPI would be active against all efflux pumps of the RND family and that EPIs demonstrated different levels

of effect on efflux mechanisms from the same organism. Aase *et al.* (2000) observed resistance to BAC by 10% of the *L. monocytogenes* strains they tested. They observed that the MIC of isolates sensitive to BAC and EtBr ( $BC^S EB^S$ ) was almost the same as resistant strains ( $BC^R EB^R$ ) following adaptation from 1 - 2  $\mu\text{g ml}^{-1}$  to 6 - 7  $\mu\text{g ml}^{-1}$  BAC, which remained stable for 7 days. Their study involved strains with different phenotypes and while they observed efflux of EtBr from the organisms that were both resistant and adapted to BAC and EtBr, strains that were BAC resistant and EtBr sensitive ( $BC^R EB^S$ ) did not demonstrate the same level of resistance following adaptation. This they suggested was due to another mechanism, which may be linked to the already present BAC resistance mechanism. Soumet *et al.* (2005) agreed with the work by Aase *et al.* (2000) as they observed 42% of *L. monocytogenes* strains isolated from food and food processing environments demonstrated low susceptibility to BAC with MICs of 10 – 15  $\mu\text{g ml}^{-1}$  compared to susceptible strains with MICs of 2.5 – 3.75  $\mu\text{g ml}^{-1}$ . Studies showed that another mechanism apart from efflux was present in  $BC^R EB^S$  strains, which they did not attribute to plasmids

#### **4.2.1 *L. monocytogenes* conclusion**

*L. monocytogenes* becomes less susceptible to BAC following treatment with all of the anionic detergents but not with the non-ionic detergent suggesting that it is the charge on the detergent molecule that is influencing susceptibility. This is supported by efflux studies that show SAS causes greater efflux of EtBr and overrides the effect of EPIs. Overall the results strongly suggest that the anionic detergents influence an efflux mechanism in *L. monocytogenes*.

As seen with *E. coli*, *L. monocytogenes* becomes more susceptible to NaDCC following treatment with all of the detergents demonstrating an enhanced oxidising action of NaDCC.



This research has shown that commercially used detergents can influence the sensitivity of pathogenic food borne microorganisms to BAC and NaDCC and the effects have been seen to differ between *E. coli* and *L. monocytogenes*.

The fact that some detergents are causing a reduction in susceptibility to disinfectant may be a major factor in persistent resident organisms in a food-processing environment that could impact further up the food chain to the home and other food preparation environments. The choice of detergents and disinfectants used in food industry cleaning procedures should therefore be carefully considered, as should rotation of cleaning products to avoid development of resistance.

It is recognised that in this study a lower than in use concentration of disinfectant was used but there are situations in a food-processing environment, such as drains, where the disinfectant is working at below the recommended concentration. Resistance to sub lethal concentrations could lead to adaptation and cross-resistance to other disinfectants. However, resistance to NaDCC is unlikely as all detergents caused an increase in susceptibility.

In contrast, the observed increase in susceptibility of *E. coli* demonstrated that some detergents enhanced the effect of the disinfectant suggesting that different combinations of detergents and disinfectants could be optimised which may reduce cost and impact on the environment.

Overall the results have shown that there is no simple explanation for the observed changes in susceptibility but a combination of interacting factors with more than one factor involved and, is very much species dependent.

This study has shown that isolation of persistent problematic organisms from a factory environment can be studied to identify the most effective detergent – disinfectant combinations. Such combinations can then be used to maximise the chance of eradication of said organisms from the factory.

Further work at molecular level is required to determine the action of the surfactants deep within the membrane, particularly where a reduction in susceptibility to disinfectant was observed, as *L. monocytogenes* is an organism of great concern to the food industry. This would involve investigations into whether the charge on the detergents influenced induction of efflux through analysis of gene expression of treated cells. This would also help in understanding why *L. monocytogenes* continues to uptake EtBr following addition of glucose. For *E.coli* radiolabelling of detergents would enable tracking through the cell envelope and a view into the effects of the detergents.

Studies of LPS could be performed to determine whether the detergents are behaving as chelating agents and disrupting the bonds that maintain its structure.

Susceptibility studies should be repeated with the addition of food soil, to simulate the food-processing environment, and to establish the effect of soil on the detergent-disinfectant combination. Investigations into the effects on biofilms should also be undertaken as organisms in biofilms are known to be less susceptible to the action of disinfectants than cells in suspension.

This study looked at the effects of detergents on disinfectant susceptibility of only two organisms, which should now be extended to other persistent organisms in food processing environments. The effects of a wider range of disinfectants on susceptibility other organisms following detergent treatment should also be investigated.

## **7 Posters presented at conferences and publication**

### **Poster presentations**

Walton, J.T. (2006) Investigation into the mechanisms of detergent induced changes in disinfectant susceptibility. Society of General Microbiology, University of York. 11-14<sup>th</sup> September, 2006.

Walton, J.T., Hill D.J., Protheroe, R.G., Hayes, R., Neville, A. and Gibson, H. (2010) Investigation into the mechanisms of detergent induced changes in disinfectant susceptibility of *Listeria monocytogenes*. International Symposium on problems of Listeriosis. Porto, Portugal. 5<sup>th</sup> May 2010.

### **Publication**

Walton, J.T., Hill, D.J., Protheroe, R.G., Nevill, A. and Gibson. H. (2008) Investigation into the effect of detergents on disinfectant susceptibility of attached *Escherichia coli* and *Listeria monocytogenes*. *Journal of Applied Microbiology*. **105**. 309-315.

## References

- Aarnisalo, K., Salo, S., Meittinen, H., Suihko, M.L. Wirtanen, G., and Autio, T. (2000) Bacterial efficiencies of commercial disinfectants against *Listeria monocytogenes* on surfaces. *Journal of Food Safety*. **20**. 237 – 250.
- Aarnisalo, K., Lunden, J., Korkeala, H. and Wirtanen, G. (2007) Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *Swiss Society of Food Science and Technology*. **40**.1041 - 1048.
- Aase, B., Sundheim, G., Langsrud, S. and Rorvik, L.M. (2000) Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. *International Journal of Food Microbiology*. **62**. 57 - 63.
- Ahmed, M., Borsch, C.M. Taylor, S., Vazquez, N. and Neyfakh, A.A. (1994) A protein that activates expression of a multidrug efflux transporter upon binding to the transporter substrates. *Journal of Biological Chemistry*. **269**. 28506 – 28513.
- Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A. and Maier, R.M. (2000) Rhamnolipid – induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. *Applied and Environmental Microbiology*. **66**. 3262 – 3268.
- Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K. and Helander, I.M. (2000) Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*. **66**. 2001 – 2005.
- Allen, M.J., Morby, A. P. and White, G.F. (2004) Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochemical and Biophysical Research Communications*. **318**. 397 - 404.
- Allison, D.G. (1998) Exopolysaccharide production in bacterial biofilms [online]. [Accessed 27 March 2012]. Available at <<http://hdl.handle.net/1807/82>>
- Allison, D.G. and Matthews, M.J. (1992) Effect of polysaccharide interactions on antibiotic susceptibility of *Pseudomonas aeruginosa*. *Journal of Applied Microbiology*. **73**. 484 – 488.
- Allison, D. and Gilbert, P. (2004) Bacteria IN *Hugo & Russell's Pharmaceutical Microbiology* 7<sup>th</sup> Ed. EDS Denyer, S.P., Hodges, N.A. and Gorman, S.P. Blackwell Publishing Limited. Oxford.

Ananta, E., Heinz, V. and Knorr, D. (2004) Assessment of high pressure induced damage on *Lactobacillus rhamnosus* GG by flow cytometry. *Food Microbiology*. **21**. 567 – 577.

Anon (1997) BS EN 1276:1997 Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas – Rest method and requirements (phase 2, step 1).

Ayres, H. M., Furr, J. R. and Russell A. D. (1999) Effect of permeabilizers on antibiotic sensitivity of *Pseudomonas aeruginosa*. *Letters in Applied Microbiology*. **28**. 13 – 16.

Babajide, O., Okandeji, D.M., Greenwald, J.W. and Sello, J.K. (2011) Synthesis and evaluation of inhibitors of bacterial drug efflux pumps of the major facilitator superfamily. *Bioorganic and Medicinal Chemistry*. **19**. 7679 – 7689.

Bagge-Raven, D., Ng, Y., Hjelm, M., Christiansen, J.N., Johansen, C. and Gram, L. (2003) The microbial ecology of processing equipment in different fish industries – analysis of microflora during processing and following cleaning and disinfection. *International Journal of Food Microbiology*. **87**. 239 – 250.

Bar-Ness, R., Avrahamy, N., Matsuyama, T. and Rosenberg, M. (1988) Increased cell hydrophobicity of a *Serratia marcescens* NS38 mutant lacking wetting activity. *Journal of Bacteriology*. **170**. 4361 – 4364.

Begley, M., Gahan, C.G.M. and Hill, C. (2002) Bile stress in *Listeria monocytogenes* LO28: adaptation, cross-protection and identification of genetic loci involved in bile resistance. *Applied and Environmental Microbiology*. **68**. 6005 – 6012.

Bessems, E. (1998) The effect of practical conditions on the efficacy of disinfectants. *International Biodeterioration and Biodegradation*. **41**. 177 – 183.

Best, M., Kennedy, M.E. and Coates, F. (1990) Efficacy of disinfectants against *Listeria* spp. *Applied and Environmental Microbiology*. **56**. 377 - 380.

Bhairi, S.M. Ph.D. and Mohan, C. Ph.D. (2007) *Detergents: A guide to the properties and uses of detergents in biological systems*. Merck Chemicals, Germany.

Bhattachatya, S. and Mandal, S. S. (1997) Role of hydrophobic effect and surface charge in surfactant-DNA association. *Indian Journal of Biochemistry and Biophysics*. **34**. 11 - 7.

Bhavsar, A.P., Erdman, L.K., Schertzer, J.W. and Brown, E.D. (2004) Teichoic acid is an essential polymer in *Bacillus subtilis* that is functionally distinct from teichuronic acid. *Journal of Bacteriology*. **186**. 7865 – 7873.

Bina, X.R., Provenzano, D., Nguyen, N. and Bina, J.E. (2008) *Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. *Infection and Immunity*. **76**: 3595–3605

Blackman, I.C. and Frank, J.F. (1996). Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *Journal of Food Protection*. **59**. 827 - 831.

Bloomfield, S.F. and Miles, G.A. (1979) The antibacterial properties of sodium dichloroisocyanurate and sodium hypochlorite formulations. *Journal of Applied Bacteriology*. **46**. 65 – 73.

Bohnert, J.A. Karamian, B. and Nikaido, H. (2010) Optimized Nile Red efflux assay of AcrAB–TolC multidrug efflux system shows competition between substrates. *Antimicrobial Agents and Chemotherapy*. **54**. 3770 – 3775.

Bond, P.J., Cuthbertson, J. and Sansom, M.S.P. (2005) Simulation studies of the interactions between membrane proteins and detergents. *Biochemical Society Transactions*. 33, part 5.

Borges – Walmsley, M.I. and Walmsley, A.R. (2001) The structure and function of drug pumps. *Trends in Microbiology*. **9**. 71 – 79.

Boulangue-Petermann, L., Bellon-Fontaine, M.N. and Baroux, B. (1995) An electrochemical method for assessing biodeposition on stainless steel. *Journal of Microbiological Methods*. **32**. 83 – 96.

Boulangue - Petermann, L. (1996) Processes of bioadhesion on stainless steel surfaces and cleanability: A review with special reference to the Food Industry. *Biofouling*. **10**. 275 - 300.

Braoudaki, M. and Hilton, A.C. (2005) Mechanisms of resistance in *Salmonella enterica* adapted to erythromycin, benzalkonium chloride and triclosan. *International Journal of Antimicrobial Agents*. **25**. 31 – 37.

Briandet, R., Leriche, V., Carpentier, B. and Bellon-Fontaine, M.N. (1999) Effects of growth procedure on the surface hydrophobicity of *Listeria monocytogenes* cells and their adhesion to stainless steel. *Journal of Food Protection*. **62**. 994 – 998.

Brown, D.G. (2005) Effects of non-ionic surfactants on the cell surface hydrophobicity and apparent haymaker constant of a *Sphingomonas* sp. *Environmental Science and Technology* **40**. 195 – 201.

Brown, M.R.W. and Richards, R.M.E. (1964) Effect of Polysorbate (Tween) 80 in the resistance of *Pseudomonas aeruginosa* to chemical inactivation. *Journal of Pharmacy and Pharmacology*. **16**, 51T-55T.

Brown, M.R.W. and Winsley, B.E. (1969) Effect of Polysorbate 80 on cell leakage and viability of *Pseudomonas aeruginosa* exposed to rapid changes of pH, temperature and tonicity. *Journal of General Microbiology*. **56**. 99 – 107.

Brown, M.R.W. and Winsley, B.E. (1971) Synergism between polymyxin and Polysorbate 80 against *Pseudomonas aeruginosa*. *Journal of General Microbiology*. **68**, 367 – 373.

Cairney, J.W.G. and Smith, S.E. (1993) The influence of monovalent cations on efflux of phosphate from ectomycorrhizal basidiomycete *Pisolithus tinctorius*. *Mycological Research*. **97**. 1267 – 1271.

Caprioli, A., Tozzi, A. E., Rizzoni, G. and. Karch, H. (1997) Non-O157 Shiga toxin-producing *Escherichia coli* infections in Europe. *Emerging Infectious Diseases*. **3**. 578 – 579.

Carpentier, B. and Cerf, O. (1993) Biofilms and their consequences with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology*. **75**. 499 - 511.

Cerda-Maira, F.A., Ringelberg, C.S. and Taylor, R.K. (2008) The bile response repressor BreR regulates expression of the *Vibrio cholerae* breAB efflux system operon. *Journal of Bacteriology*. **190**: 7441–7452.

Cerf, O., Carpentier, B, and Sanders, P (2010) Tests for determining in – use concentrations of antibiotics and disinfectants are based on entirely different concepts: “Resistance” has different meanings. *International Journal of Food Microbiology*. **136**. 247 – 254.

Chapman, J.S. (1998) Characterising bacterial resistance to preservatives and disinfectants. *International Biodeterioration and Biodegradation*. **41**. 241 - 245.

Chapman, J.S. (2003) Disinfectant mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation*. **51**. 271 – 276.

Chapman, J.S., Diehi, M.A. and Lyman, R.C. (1993) Biocide susceptibility and intracellular glutathione in *Escherichia coli*. *Journal of Medical Microbiology*. **12**, 403 – 407.

Chatterjee, A., Chaudhuri, S., Saha, G., Gupta, S, and Chowdhury, R. (2004) Effect of bile on the cell surface permeability barrier and efflux system of *Vibrio cholerae*. *Journal of Bacteriology*. **186**: 6809–6814.



Clark, W.B., Lane, M.D., Beem, E., Bragg, S.L. and Wheeler, T.T. (1985) Relative hydrophobicities of *Actinomyces viscosus* and *Actinomyces naeslundii* and their adsorption to saliva treated hydroxyapatite. *Infection and Immunity*. **47**. 730 - 736.

Clasen, T. and Edmondson, P. (2006) Sodium dichloroisocyanurate (NaDCC) tablets as an alternative to sodium hypochlorite for the routine treatment of drinking water at the household level. *International Journal of Hygiene and Environmental Health*. **209**. 173 – 181.

Cloete, T.E. (2003) Resistance mechanisms of bacteria to antimicrobial compounds. *International Biodeterioration and Biodegradation*. **51**. 277 – 282.

Coates, D. (1977) Kelsey-Sykes capacity test: origin, evolution and current status. *Pharmaceutical Journal*. **219**. 402 - 403.

Cordier, J.L., Putallaz, T. and Cox, L.J. (1989) Impedimetric determination of activity of disinfectants on *Listeria* : Preliminary study. *International Journal of Food Microbiology*. **8**. 293 – 297.

Cortez-Cordova, J. and Kumar, A. (2011) Activity of the efflux pump inhibitor phenylalanine-arginine  $\beta$ -naphthylamide against the AdeFGH pump of *Acinetobacter baumannii*. *International Journal of Antimicrobial Agents*. **37**. 420 – 424.

Costerton ,J.W. (1995) Overview of microbial biofilms. *Journal of Industrial Microbiology and Biotechnology*. **15**. 137 – 140.

Delcour, A.H. (2009) Outer membrane permeability and antibiotic resistance. *Biochemica and Biophysica Acta*. **1794**. 808 – 816.

Denyer, S.P. and Stewart, G.S.A.B. (1998) Mechanisms of action of disinfectants. *International Biodeterioration and Biodegradation*. **41**. 261 - 268.

Denyer, S.P. and Maillard, J.-Y. (2002) Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *Journal of Applied Microbiology Symposium Supplement*. **92**. 35S - 45S.

Dhir, V.K. and Dodd, C.E. (1995) Susceptibility of suspended and surface attached *Salmonella enteritidis* to biocides and elevated temperatures. *Applied and Environmental Microbiology*. **61**. 1731 – 1738.

Dukan, S., Belkin, S. and Touati, D. (1999) Reactive species are partially involved in the bactericidal action of hypochlorous acid. *Archives of Biochemistry and Biophysics*. **367**. 311 – 316.

Dundas, S., Todd, W.T.A., Stewart, A.I., Muddoch, P.S., Chaudari, A.K.R. and Hutchinson, S.J. (2001) The Central Scotland *Escherichia coli* O157:H7 Outbreak: Risk Factors for the Hemolytic Uremic Syndrome and Death among Hospitalized Patients. *Clinical Infectious Diseases*. **33**. 923 - 931.

Dychdala, G.R. (2001) Chlorine and Chlorine Compounds. in Block, S.S.(ed.) Disinfection, Sterilization and Preservation. 5<sup>th</sup> Edition. Lippincott Williams and Wilkins, Philadelphia. pp 135 - 158.

Earnshaw, A.M. and Lawrence, L.M. (1998) Sensitivity to commercial disinfectants, and the occurrence of plasmids within various *Listeria monocytogenes* genotypes isolated from poultry products and the poultry processing environment. *Journal of Applied Microbiology*. **84**. 642 – 648.

Eswaran, J., Koronakis, E., Higgins, M., Hughes, C. and Koronakis, V. (2004) Three's company: component structures bring a closed view of tripartite drug efflux pumps. *Current Opinion in Structural Biology*. **14**. 741 – 747.

Fisher, J. (2003) Cleaning procedures in the factory / Types of disinfectant IN Trugo, L. and Finglas, P.M. editors, *Encyclopaedia of Food Sciences and Nutrition*. Elsevier Science Ltd. 1382 – 1385.

Flahaut, S., Frere, J., Boutibonnes, P. and Auffrey, Y. (1996) Comparison of the bile salts and sodium dodecyl sulphate stress responses in *Enterococcus faecalis*. *Applied and Environmental Microbiology*. **62**. 2416 – 2420.

Fonnesbach, V.B., Huss, H.H., Ojeniyi, B., Ahrens, P. and Gram, L. (2001) Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. *Applied and Environmental Microbiology*. **67**. 2586 – 2595.

Frank, J.F. and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection*. **53**. 550 - 554.

Frank, J.F. (2001) Microbial attachment to food and food contact surfaces. *Advances in Food and Nutrition Research*. **43**. 320 – 370.

Galbraith, H., Miller, T.B., Paton, A.M. and Thompson, J.K. (1971) Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. *Journal of Applied Bacteriology*. **34**. 803 – 813.

Ganesh, K.C. and Anand, S.K. (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*. **42**: 9 - 27.

Garcia – Saez, A.J. and Schwille, P. (2010) Surface analysis of membrane dynamics. *Biochemica et Biophysica Acta*. 766 – 776.

Gardner, J.F. and Peel, M.M. (2001) *Sterilization, Disinfection and Infection Control*. Third Edition. Australia: Southwood Press Pty Limited.

Gelinas, P and Goulet, J. (1983) Neutralization of the activity of eight disinfectants by organic matter. *Journal of Applied Microbiology*. **54**.243 – 247.

Gibson, H., Taylor, J.H., Hall, K.E. and Holah, J.T. (1999) Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology*. **87**. 41 - 48.

Gilbert, P., Das, J.R. and Allison, D.G. (2001) Assessment of resistance towards biocides following the attachment of micro organisms to, and growth on, surfaces. *Journal of Applied Microbiology*. **91**. 248 – 254.

Gilbert, P., Jones, M.V., Allison, D.G., Heys, S., Maira, T. and Wood, P. (1998) The use of polyoxmer hydrogels for the assessment of biofilm susceptibility towards biocide treatments. *Journal of Applied Microbiology*. **85**. 985 – 990.

Gilbert, P. and Mc Bain, A. J. (2003) Potential impact of increased use of biocides in consumer products on prevalence of anti biotic resistance. *Clinical Microbiology Reviews*. **16** . 189 – 208.

Glover, R.E., Smith, R.R., Jones, M.V., Jackson, S.K. and Rowlands, C.R. (1999) An EPR investigation of surfactant action on bacterial membranes. *FEMS Microbiology Letters*. **177**, 57 – 62.

Godreuil, S., Galimand, M., Gerbaud, G., Jacquet, C. and Courvalin, P. (2003) Efflux pump Lde is associated with fluoroquinolone resistance in *Listeria monocytogenes*. *Antimicrobial Agents and Chemotherapy*. **47**. 704 – 708.

Gomez-Escalada, M., Russell, A.D. Maillard, J.Y. and Ochs, D. (2005) Triclosan-bacteria interactions: single or multiple target sites? *Letters in Applied Microbiology*. **41**. 476 – 481.

Gorman, S. and Scott, E. (2004) Chemical disinfectants, antiseptics and preservatives IN Hugo & Russell's Pharmaceutical Microbiology 7<sup>th</sup> Ed. EDS Denyer, S.P., Hodges, N.A. and Gorman, S.P. Blackwell Publishing Limited. Oxford.

Gram, L., Bagge-Ravn, D., Yin Ng, Y., Gymoese, P. and Fonnesbech, V.B. (2006) Influence of food spoilage matrix on cleaning and disinfection efficacy on food surface attached *Listeria monocytogenes*. *Food Control*. **18**. 1165 – 1171.

Hamilton, W.A. (1971) Membrane-active antibacterial compounds. In Hugo, W.B. (ed.) *Inhibition and Destruction of the microbial cell*, London: Academic Press.

Hammond, S.M., Lambert, P.A. and Rycroft, A.N. (1984) Walls of Gram-positive bacteria, pp 29 - 56. In *The bacterial cell surface*. Kapitan Szabo Publishers, Washington, DC.

Hancock, R.E.W. and Chapple, D.S. (1999) Peptide Antibiotics. *Antimicrobial Agents and Chemotherapy*. **43**, 1317 - 1323.

Hayes, R.C. (2006) Interaction of Detergents and Disinfectants upon surface adhered populations of *Escherichia coli* and *Listeria monocytogenes*. PhD. Thesis, University of Wolverhampton.

Health Protection Agency (HPA) (2012) A-Z Epidemiological Data [online]. [Accessed 23 March 2012]. Available at <<http://www.hpa.org.uk/Topics/InfectiousDiseases>>

Heinzel, M. (1988) The phenomena of resistance to disinfectants and preservatives. In Payne, K.R. (ed.) *Critical Reports in Applied Chemistry. Industrial Biocides* Volume 23. John Wiley & Sons, pp.52 - 62.

Heir, E., Sundheim, G. and Holck, A.L. (1995) Resistance to quaternary ammonium compounds in *Staphylococcus* spp. isolated from the food industry and nucleotide sequence of resistant plasmid pST 827. *Journal of Applied Bacteriology*. **79**. 149 - 156.

Heir, E., Sundheim, G. and Holck, A.L. (1998) The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. *FEMS Microbiology Letters*. **163**. 49 - 56

Heir, E., Sundheim, G. and Holck, A.L. (1999) The qacG gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. *Journal of Applied Microbiology*. **86**. 378 – 388.

Heir, E., Lindstedt, B-A., Rotterud, O-J., Vardund, T., Kapperud, G. and Nesbakken, T. (2004) Molecular epidemiology and disinfectant susceptibility of *Listeria monocytogenes* from meat processing plants and human infections. *International Journal of Food Microbiology*. **96**. 85 – 96.

Helander, I.M. and Mattila-Sandholm, T. (2000) Fluorimetric analysis of Gram-negative bacterial permeabilization. *Journal of Applied Microbiology*. **88**. 213 – 218.

Helke, D.M., Somers, E.B. and Wong, A.C.L. (1993) Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and buna-N in the presence of milk and individual milk components. *Journal of Food Protection*. **56**. 479 - 484.

- Holah, H., Betts, R.P. and Thorpe, R.H. (1989) The use of epifluorescence microscopy to determine surface hygiene. *International Biodeterioration*. **25**. 147 – 153.
- Holah, J. (1995) Disinfection of food production areas. *Revue Scientific et Technique Office*. **14**. 343 – 363.
- Holah, J.T., Taylor, J.H., Dawson, D.J. and Hall, K.E. (2002) Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *Journal of Applied Microbiology Symposium Supplement*, **92**, 111S - 120S.
- Hood, S.K. and Zottola, E.A. (1995) Biofilms in food processing. *Food Control*. **6**. 9 – 18.
- Hood, S.K. and Zottola, E.A. (1997) Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology*. **37**. 145 – 153.
- Hugo, W.B. (1971) Inhibition and destruction of the microbial cell. Academic Press. London.
- Hugo, W.B., Denyer, S.P., Hodges, N.A. and Gorman, S.P. (2004) *Hugo and Russell's Pharmaceutical Microbiology*. Blackwell Science.
- Im, S.H., Jeong, Y.H. and Ryoo, J.J. (2008) Simultaneous analysis of anionic, amphoteric, non-ionic and cationic surfactant mixtures in shampoo and hair conditioner by RP-HPLC/ELSD and LC/MS. *Analytica Chimica Acta*. **619**. 129 – 136.
- Ishikawa, S., Matsumara, Y., Yoshizako, F. and Tsuchido, T. (2002) Characterization of a cationic surfactant – resistant mutant isolated spontaneously from *Escherichia coli*. *Journal of Applied Microbiology*. **92**. 261 – 268.
- Jarlier, V. and Nikaido, H. (1994) Mycobacterial cell wall; structure and role in natural resistance to antibiotics. *FEMS Microbiology Letters*. **123**. 11 – 18.
- Jones, M.V., Herd, T.M. and Christie, H.J. (1989) Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. *Microbios*. **58**, 49 - 61.
- Jones, I.G. and Midgley, M. (1985) Expression of a plasmid borne ethidium resistance determinant from *Staphylococcus* in *Escherichia coli*: evidence for an efflux system. *FEMS Microbiology Letters*. **28**. 355 – 358.
- Jordan, S., Hutchings, M.I. and Thorsten, M. (2008) Cell envelope stress response in Gram – positive bacteria. *FEMS Microbiological Reviews*. **32**. 107 – 146.

- Joynson, J.A., Forbes, B. and Lambert, R.J.W. (2002) Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. *Journal of Applied Microbiology*. **93**, 96 – 107.
- Kabara, J.J. (1978) Structure-function relationships of surfactants as antimicrobial agents. *Journal of the Society of Cosmetic Chemists*. **29**, 733 - 741.
- Karmali, M.A. (1989) Infection by verocytotoxin *Escherichia coli*. *Clinical Microbiology. Revue*. **2**. 15 – 38.
- Kazama, H., Hamashima, H., Sasatsu, M. and Arai, T. (1998) Distribution of the antiseptic resistance gene qac Edelta 1 in Gram – positive bacteria. *FEMS Microbiology Letters*. **165**. 295 – 299.
- Kim, Y.M., Farrah, S. and Baney, R.H. (2007) Membrane damage of bacteria by silanols treatment. *Electronic Journal of Biotechnology*. **10**. 252 – 259.
- Kiss, R., Tirczka, T., Szita, G., Bernath, S. and Gyorgy, C. (2006) *Listeria monocytogenes* food monitoring data and incidence of human listeriosis in Hungary, 2004. *International Journal of Food Microbiology*. **112**. 71 – 74.
- Kleanthous, H., Smith, H.R., Scotland, S. M., Gross, R.J., Rowe, B., Taylor, C.M. and Milford, D.V. (1990) Haemolytic – uraemic syndromes in the British Isles, 1985 – 8: association with verocytotoxin producing *Escherichia coli*. Part 2: Microbiological aspects. *Archives of Disease in Childhood*. **65**. 722 – 7.
- Knight, G.C. and Craven, H.M. (2010) A model system for evaluating surface disinfection in dairy factory environments. *International Journal of Food Microbiology*. **137**. 161 – 167.
- Koike, R., Kitagawa, F. and Otsuka, K. (2007) Simultaneous determination of amphoteric surfactants in detergents by capillary electrophoresis with indirect UV detection. *Journal of Chromatography*. **1139**. 136 – 142.
- Kramer, V.C. and Nickerson, K.W. (1984) Prevalence of extreme detergent resistance among the *Enterobacteriaceae*. *Canadian Journal of Microbiology*. **30**, 711 – 713.
- Krysinski, E.P., Brown, L.J. and Marchiesello, J. (1992) Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection*. **55**, 246 - 251.
- Kuda, T., Yano, T and Kuda, M.T. (2007) Resistance to benzalkonium chloride of bacteria dried with food elements on stainless steel surface. *Swiss Society of Food Science and Technology*. **41**, 9888 – 993.

Kuda, T., Iwase, T., Yuphakhun, C., Takahashi, H., Koyanagi, T. and Kimura, B. (2011) Sufactant-disinfectant resistance of *Salmonella* and *Staphylococcus* adhered and dried on surfaces with egg compounds. *Food Microbiology*. **28**. 921 – 925.

Kumar, C.G. and Anand, S.K. (1998) Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*. **42**. 9 – 27.

Lambert, P.A. (2004) Mechanisms of action of biocides. IN Fraise, A.P., Lambert, P.A. and Maillaird, J-Y (eds.) *Russell, Hugo and Aycliffe's Principles and Practice of Disinfection, Preservation and Sterilization*. Blackwell Publishing Ltd. pp 139 - 148.

Lambert, P.A. and Hammond, SM. (1973) Potassium fluxes. First indications of membrane damage in microorganisms. *Biochemical and Biophysical Research*. **54**. 796 – 799.

Lambert, P. (2004) Mechanisms of action of antibiotics and synthetic anti-infective agents. IN Hugo & Russell's Pharmaceutical Microbiology 7<sup>th</sup> Ed. EDS Denyer, S.P., Hodges, N.A. and Gorman, S.P. Blackwell Publishing Limited. Oxford.

Lambert, P. and Le Pecq, J.B. (1984) Effect of mutation, and metabolic inhibitors on the accessibility of nucleic acids to ethidium bromide in *Escherichia coli* cells. *Biochemistry*. **23**. 166 – 176.

Langsrud, S. and Sundheim, G. (1997) Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound. *Journal of Applied Microbiology*. **82**. 705 – 712.

Langsrud, S., Sundheim, G. and Borgmann-Strahsen, R. (2003) Intrinsic and acquired resistance to quaternary ammonium compounds in food-related *Pseudomonas* spp. *Journal of Applied Microbiology*. **95**. 874 – 882.

Langsrud, S., Sundheim, G. and Holck, A.L. (2004) Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. *Journal of Applied Microbiology*. **96**, 201 – 208.

Leelaporn, A., Paulsen, I.T., Tennent, J.M. Littlejohn, T.G. and Skurray, R.A. (1994) Multidrug resistance to antiseptics and disinfectants in coagulase-negative staphylococci. *Journal of Medical Microbiology*. **40**. 214 – 220.

Lehmann, R.H. (1988) Synergisms in disinfectant formulations. In Payne, K.R. (ed.) Critical Reports in Applied Chemistry. *Industrial Biocides Volume 23*. John Wiley & Sons, pp.68-88.

Levy, S.B. (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. *Journal of Applied Microbiology Symposium Supplement*. **92**. 65S – 71S.

Lewis, J.J. (1995) An introduction to surfactants IN Preservation of surfactant formulations Morpeth, F.F.(ed). Blackie Academic Professional. pp 53 - 82

Levin, B.R. and Rozen, D.E. (2006) Non-inherited antibiotic resistance. *National Revue of Microbiology*. **4**, 556 – 562.

Li, X-Z., Livermore, D.M. and Nikaido, H. (1994) Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol and norflaxin. *Antimicrobial Agents and Chemotherapy*. **38**. 1732 – 1741.

Li, X-Z. and Nikaido, H. (2009) Efflux-mediated drug resistance in bacteria: an update. *Drugs*. **69**: 1555 – 1623.

Li, J. and McLandsborough, L.A. (1999) The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *International Journal of Food Microbiology*. **53**. 185 – 193.

Lindsey, D. and von Holy, A. (1997) Evaluation of dislodging methods for laboratory – grown bacterial biofilms. *Food Microbiology*. **14**. 383 – 390.

Lomovskaya, O., Warren, M.S., Lee, A., Gallazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Leger, R., Hecker, S., Watkins, W., Hoshino, K., Ishida, H. and Lee, V.J. (2001) Identification and characterisation of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrobial Agents Chemotherapy*. **45**. 105 – 116.

Lorinczy. D., and Kocsis. B (2001) Interaction between lipopolysaccharide and detergents detected by differential scanning calorimetry. *Thermochimica Acta*. 372(1-2). 19 – 23.

Lourenco, A., Neves, E. and Brito, L. (2009) Susceptibility of *Listeria monocytogenes* from traditional cheese – dairies to in use sanitizers. *Food Control*. **20**. 585 – 589.

Lukac, M., Lacko, I., Bukovsky, M., Kyselova, Z., Karlovska, J., Horvath, B. and Devinsky, F. (2010) Synthesis and antimicrobial activity of a series of optically active quaternary ammonium salts derived from phenylalanine. *Central European Journal of Chemistry*. **8**. 194 – 201.

Lunden, J., Autio, T., Markkula, A., Hellstrom, S. and Korkeala, H. (2003) Adaptive and cross - adaptive response of persistent and non – persistent *Listeria monocytogenes* strains to disinfectants. *International Journal of Food Microbiology*. **82**, 265 - 272.

Lyon, B.R. and Skurray, R.A. (1987) Antimicrobial resistance of *Staphylococcus aureus*: Genetic basis. *Microbial Reviews*. **51**. 88 – 134.



- McDonnell, G. and Russell, A.D. (1999) Antiseptics and disinfectants: activity, action and resistance. *Clinical Microbiology Reviews*. **12**. 147-179.
- McMurray, L.M., Oethinger, M. and Levy, S.B. (1998) Over – expression of *marA*, *soxS*, or *acrB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiology Letters*. **166**. 305 – 309.
- Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H. and Hearst, J.E. (1995) Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Molecular Microbiology*. **16**. 45 – 55.
- Mafu, A.A., Roy, D, Goulet, J., Magny, P. (1990) Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact times. *Journal of Food Protection*. **53**, 742 - 746.
- Mafu, A. A., Roy, D., Goulet, J. and Savoie, L. (1991) Characterization of physio-chemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. *Applied and Environmental Microbiology*. **57**. 1969 – 1973.
- Maillard, J.Y. (2002) Bacterial target sited for biocide action. *Journal of Applied Microbiology Symposium Supplement*. **92**, 16S – 27S.
- Maillard, J.Y. (2007) Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? *Journal of Hospital Infection*. **65**. 60 – 72.
- Marcotte, L., Barbeau, J., Edwards, K., Karlsson, G. and Lafleur, M. (2005) Influence of the lipid composition on the membrane affinity and the membrane perturbing ability of cetylpyridinium chloride. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. **8**. 194 – 201.
- Markham, P.N. and Neyfakh, A.A. (2001) Efflux – mediated drug resistance in Gram – positive bacteria. *Current Opinion in Microbiology*. **4**, 509 – 514.
- Marquez, B. (2005) Bacterial efflux systems and efflux pumps inhibitors. *Biochimie*. **87**. 1137-1147.
- Martins, A., Machado, L., Costa, S., Cerca, P., Spengler, G., Viveiros, M. and Amaral, L. (2011) Role of calcium in the efflux system of *Escherichia coli*. *International Journal of Antimicrobial Antibiotics*. **37**. 410 – 414.
- Martinez, J.L. and Rojo, F. (2011) Metabolic regulation of antibiotic resistance. *FEMS Microbiology Reviews*. **3**. 768 – 789.
- Mata, M.T., Baqueroa, F. and Perez-Diaz, J.C. (2000). A multidrug efflux transporter in *Listeria monocytogenes*. *FEMS Microbiology Letters*. **187**. 185 - 188.

Mazzola, P.G., Vessoni Penna, T.C., da S Martins, A.M. (2003) Determination of decimal reduction time (*D* value) of chemical agents used in hospitals for disinfection purposes. *BMC Infectious Diseases*. **3**. 24 - 34

Mechin, L., DuBois-Brissonet, F., Heyd, B. and Leveau, J.Y. (1999) Adaptation of *Pseudomonas aeruginosa* ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. *Journal of Applied Microbiology*. **86**. 859 - 866.

Mereghetti, L., Quentin, R., Marquet - Van Der Mee, N., and Audurier, A (2000) Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. *Applied and Environmental Microbiology*. **66**. 5083 – 5086.

Meyer, B. (2006) Does microbial resistance to biocides create a hazard to food hygiene? *International Journal of Food Microbiology*. **112**, 275 - 279.

Middleton, K.E. (2003) Investigation of the effect of acid and detergents on the biocide susceptibility of *Escherichia coli* 0157:H7. PhD Thesis. University of Wolverhampton.

Midgley, M. (1986) The phosphodiuion ion efflux system of *Escherichia coli*: relationship to the ethidium efflux system and energetic studies. *Journal of General Microbiology*. **132**. 3187 - 3193.

Moore, S.L. and Payne, D.N. (2004) Types of antimicrobial agents. in Fraiese, A.P., Lambert, P.A. and Maillaird, J-Y (eds.) Russell, Hugo and Aycliffe's *Principles and Practice of Disinfection, Preservation and Sterilization*. Blackwell Publishing Ltd. pp 8 - 77.

Moore, S.L., Denyer, S.P., Hanlon, G.W., Olliff, C.J., Lansley, A.B., Rabome, K. and Jones, M. (2006) Alcohol ethoxylates mediate their bacteriostatic effect by altering the cell membrane of *Escherichia coli* NCTC 8196. *International Journal of Antimicrobial Products*. **28**. 503 – 513.

Morelli, J.J. and Szajer, G. (2000) Analysis of surfactants: Part 1. *Journal of Surfactants and Detergents*. **3**. 539 - 552.

Mosteller, T.M. and Bishop, J.R. (1993) Sanitizer efficacy against attached bacteria. *Journal of Food Protection*. **56**. 34 - 41.

Muller, S., Ullrich, S., Losche, A., Loffhagan, N. and Babel, W. (2000) Flow cytometric techniques to characterise physiological states of *Acinetobacter calcoaceticus*. *Journal of Microbiological Methods*. **40**. 67 – 77.

Mustapha, A. and Liewen, M.B. (1989) Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. *Journal of Food Protection*. **52**. 306 - 311.

- Narayan, M., Welker, E., Wedemeyer, W.J. and Scheraga, H.A. (2000) Oxidative folding of proteins. *Accounts of Chemical Research*. **33**, 805 - 812.
- Nikaido, H. (1985) Role of permeability barriers in resistance to  $\beta$ -lactam antibiotics. *Pharmacology and Therapeutics*. **27**. 197 – 231.
- Nikaido, H. (1996a) Outer Membrane IN Neidhart F.C. (ed) *Escherichia coli* and *Salmonella*, Cellular and Molecular biology, ASM Press, Washington, DC. 29 – 47.
- Nikaido, H. (1996b) Multidrug efflux pumps of Gram-negative bacteria. *Journal of Bacteriology*. **178**, 5853 – 5859.
- Nikaido, H. and Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews*. **49**. 1 - 32.
- Nikaido, H. and Pages, J.M. (2012) Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiological Reviews*. **36**. 340 – 363.
- Nixdorff, K., Gmeiner, J. and Martin, H.H. (1978) Interaction of lipopolysaccharide with detergents and its possible role in the detergent resistance of the outer membrane of Gram-negative bacteria. *Biochemica et Biophysica Acta*. **510**. 87 – 98.
- Norwood, D.E. and Gilmour, A. (2000) The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multi species biofilm. *Journal of Applied Microbiology*. **88**, 512 – 520.
- Notermans, S., Dormans, J.A.M.A. and Mead, G.C. (1991) Contribution of surface attachment to the establishment of microorganisms in food processing plants: A review. Biofouling: *The Journal of Bioadhesion and Biofilm Research*. **5**. 21 – 36.
- Pages, J-M., Masi, M. and Barbe, J. (2005) Inhibitors of efflux pumps in Gram – negative bacteria. *TRENDS in Molecular Medicine*. **8**. 382 – 389.
- Palsdottir, H. and Hunte, C. (2004) Lipids in membrane protein structures. *Biochemica et Biophysica Acta*. **1666**. 2 - 18.
- Pannek, S., Higgins, P.G., Steinke, P., Jonas, D., Akova, M., Bohnert, J.A. Seifert, H. and Kern, W.V. (2006) Multidrug efflux inhibition in *Acinobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine- $\beta$ -naphthylamide. *Journal of Antimicrobial Chemotherapy*. **57**. 970 – 974.
- Papadimitriou, K., Pratsinis, H., Nebe-von-Caron, G., Kletsas, D. and Tsakalidou, E. (2006) Rapid assessment of the physiological status of *Streptococcus macedonicus* by flow cytometry and fluorescence probes. *International Journal of Food Microbiology*. **111**. 197 - 205.

Park, K-M and So, J-S. (2000) Altered cell surface hydrophobicity of lipopolysaccharide-deficient mutant of *Bradyrhizobium japonicum*. *Journal of Microbiological Methods*. **41**. 219 – 226.

Pasanan, P., Kalliokoski, P. and Pasanen, A - L. (1997) The effectiveness of some disinfectants and detergents against microbial activity. *Building and the Environment*. **32**. 281 - 287.

Paulsen, I.T., Chen, J., Nelson, K.E. and Saier, M.H. (2001) Comparative genomics of microbial drug efflux systems. *Journal of Molecular Microbiology and Biotechnology*. **3**. 145 – 150.

Paulsen, I.T. (2003) Multidrug efflux pumps and resistance: regulation and evolution. *Current Opinions in Microbiology*. **6**. 446 - 451

Payne, C .J. I., Petrovic, M., Roberts, R.J., Paul, A., Linnane, E., Walker, M., Kirby, D., Burgess, A., Smith, R. M. M., Cheasty, T., Willshaw, G. and Salmon, R.L. (2003) Vero Cytotoxin–Producing *Escherichia coli* O157 Gastroenteritis in Farm Visitors, North Wales. *Emerging Infectious Disease*. **9**. 526 – 530.

Piddock, L.J.V. (2006a) Clinically relevant chromosomally encoded multi drug resistance efflux pumps in bacteria. *Clinical Microbiology*. **19**. 382 – 402.

Piddock, L.J.V. (2006b) Multidrug-resistance efflux pumps - not just for resistance. *Nature Reviews, Microbiology*. **4**, 629 – 636.

Poole, K. (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *Journal of Molecular Microbiology and Biotechnology*. **3**. 255 – 264.

Poole, K. (2002) Mechanisms of bacterial biocides and antibiotic resistance. *Journal of Applied Microbiology Symposium Supplement*. **92**. 55S – 64S.

Poole, K. (2005) Efflux mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*. **56**. 20 – 51.

Poole, K. and Lomovskaya, O. (2006) Can efflux inhibitors really counter resistance? *Drug Discovery Today: Therapeutic Strategies*. **3**. 145 – 152.

Pos, K.M. (2009) Drug transport mechanism of the AcrB efflux pump. *Biochimica et Biophysica Acta*. **1794**. 782 – 793.

Rangel-Yagui, C.O., Pessoa-Jr, A. and Costa-Tavares, L. (2005) Micellar solubilization of drugs. *Journal of Pharmacy and Pharmacological Sciences*. **8**. 147 – 163.

Rivas, L., Fegan, N. and Dykes, G. (2007) Attachment of Shiga toxigenic *Escherichia coli* to stainless steel. *International Journal of Food Microbiology*. **115**. 89 – 94.

Rodrigues, L., Wagner, D., Viveiros, M., Sampaoi, D., Couto, I., Vavra, M., Kern, W.V. and Amaral, L. (2008) Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*. *Journal of Antimicrobial Chemotherapy*. **61**. 1076 – 1082.

Romonova, N.A., Wolffs, P.F.G., Brovko, L.Y. and Griffiths, M.W. (2006) Role of efflux pumps in adaptation and resistance of *Listeria monocytogenes* to benzalkonium chloride. *Applied and Environmental Microbiology*. **72**. 3498 – 3503.

Romanova, N.A., Gawande, P.V. Brovko, L.Y. and Griffiths, M.W. (2007) Rapid methods to assess sanitizing efficacy of benzalkonium chloride to *Listeria monocytogenes* biofilms. *Journal of Microbiological Methods*. **71**. 231 – 237.

Ronner, A.B. and Wong, A.C.L. (1993) Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless-steel and buna-N rubber. *Journal of Food Protection*. **56**. 750 – 758.

Rorvik, L.M., Caugant, D.A. and Yndestad, M. (1995) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology*. **25**. 19 – 27.

Rouquette, C., Harmon, J.B. and Shafer, W.M. (1999) Induction of the *mtrCDE* – encoded efflux pump system of *Neisseria gonorrhoea* requires MtrA, an AraC-like protein. *Molecular Microbiology*. **33**. 651 – 658.

Ruseka. I., Robbins, J. Lashen, E.S. and Costerton, J.W. (1982) Biocide testing against corrosion causing oil field bacteria helps control plugging. *Oil Gas Journal*. **80**. 253 - 264.

Russell, A.D. (1986) Chlorhexidine: antibacterial action and bacterial resistance. *Infection*. **14**, 212 - 215.

Russell, A.D. (1992) Introduction of Biocides into clinical practice and the impact of antibiotic – resistant bacteria. *Journal of Applied Microbiology*. **92**, 121S - 135S.

Russell, A.D. (1998) Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection*. **43**. S57 – S68.

Russell, A.D. (2001) Antimicrobial activity and resistance. in Block, S.S.(ed.) *Disinfection, Sterilization and Preservation*. 5<sup>th</sup> Edition. Lippincott Williams and Wilkins, Philadelphia. pp 32 - 56.

- Russell, A.D. (2002) Antibiotic and biocide resistance in bacteria: Introduction. *Journal of Applied Microbiology*. **92**. 1 - 3.
- Russell, A.D. (2003a) Bacterial outer membrane and cell wall penetration and cell destruction by polluting chemical agents and physical conditions. *Science Progress*. **86**. 283 - 311.
- Russell, A. D. (2003b) Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *The Lancet*. **3**, 794 – 803.
- Russell, A.D. (2004) Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *Journal of Hospital Infection*. **57**. 97 – 104.
- Russell, A.D. and Chopra, I. (1996) *Understanding Antibacterial Action and Resistance*, 2<sup>nd</sup> edn. Chichester: Ellis Horwood.
- Russell, A.D. and Gould, G.W. (1988) Resistance of Enterobacteriaceae to preservatives and disinfectants. *Journal of Applied Microbiology*. **65**. 167s – 195s.
- Russell, A.D., Hammond, S.A. and Morgan, J.R. (1986) Bacterial resistance to antiseptics and disinfectants. *Journal of Hospital Infection*. **7**. 213 - 225.
- Russell, A.D and McDonnell, G. (2000) Concentration: a major factor in studying biocidal action. *Journal of Hospital Infection*. **44**. 1 - 3.
- Sakagami, Y., Yokoyama, H., Nishimura, Y., Ose, Y. and Tashima, T. (1989) Mechanisms of resistance to benzalkonium chloride by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*. **55**. 2036 - 2040.
- Sanchez, M.B., Hernandez, A. and Martinez, L.J. (2009) *Stenotrophomonas maltophilia* drug resistance. *Future Microbiology*. **4**. 655 - 660.
- Schmitz, F-J., Fluit, A.C., Luckefahr, M., Engler, B., Hofmann, B., Verhoef, J., Heinz, H-P., Hadding, U. and Jones, M.E. (1998) The effect of reserpine, an inhibitor of multidrug efflux pumps, on the invitro activities of ciprofloxacin, sparfloxacin and moxifloxacin against clinical isolates of *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. **42**. 807 – 810.
- Schweizer, H.P. (2003) Efflux as a mechanisms of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genetics and Molecular Research*. **2**. 48 – 62.
- Shapiro, H.M. (2008) Flow cytometry of bacterial membrane potential and permeability. IN, Scott Champney, W. editor, *New antibiotic targets*, Humana Press, New Jersey.

Sikkema, J., De Bont, J.A.M. and Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews*. **59**. 201 - 222.

Singer, S.J. and Nicholson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. *Science*. **175**. 720 – 731.

Singer, M.M. and Tjeerdema, R.S. (1993) Fate and effects of the surfactant Sodium dodecyl Sulphate. *Reviews of Environmental Contamination and Toxicology*. **133**. 95 – 149.

Singh, A., Van Hamme, J.D. and Ward, O.P. (2006) Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology Advances*. **25**. 99 – 121.

Sirisattha, S., Momose, Y., Kitagawa, E. and Iwahashi, H. (2004) Toxicity of anionic detergents determined by *Saccharomyces cerevisiae* microarray analysis. *Water Research*. **38**. 61 – 70.

Skvarla, J., Kupka, D. and Turcaniova, L. (2002) A complementary study of hydrophobicity and surface charge of *Thiobacillus ferrooxidans*. The effect of ionic surfactants. *Acta Montanistica Slovaca*. **2**. 85 – 88

Smyth, C. J. Jonsson, P., Olsson, E., Soderlind, O., Rosengren, J., Hjerten, S. and Wadstrom, T. (1978) Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infection and immunity*. **22**. 462 – 472.

Sonnet, P., Izard, D. and Mullie, C. (2012) Prevalence of efflux – mediated ciprofloxacin and levofloxacin resistance in recent clinical isolated of *Pseudomonas aeruginosa* and its reversal by the efflux pump inhibitors 1–(1–naphthylmethyl)–piperazine and phenylalanine-arginine– $\beta$ –naphthylamide. *International Journal of Antimicrobial Agents*. **39**. 77 – 80.

Soumet, C., Ragimbeau, C. and Maris, P. (2005) Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production. *Letters in Applied Microbiology*. **41**. 291 – 296.

Stavri, M., Piddock, L.J.V. and Gibbons, S. (2007) Bacterial efflux pump inhibitors from natural sources. *Journal of Antimicrobial Chemotherapy*. **59**. 1247 – 1260.

Stickler, D.J. (2004) Intrinsic resistance of Gram-negative bacteria. in Fraiese, A.P., Lambert, P.A. and Maillaird, J-Y (eds.) Russell, *Hugo and Aycliffe's Principles and Practice of Disinfection, Preservation and Sterilization*. Blackwell Publishing Ltd. pp 154 - 165.

Sundheim, G., Landsrund, E., Heir, E. and Holck, A.L. (1998) Bacterial resistance to disinfectants containing quaternary ammonium compounds. *International Biodeterioration and Biodegradation*. **41**. 235 - 239.

- Sutterlin, H., Alexy, R. and Kummerer, K. (2008) The toxicity of the quaternary ammonium compounds benzalkonium chloride alone and in mixtures with other anionic compounds to bacteria in test systems with *Vibrio fischeri* and *Pseudomonas putida*. *Ecotoxicology and Environmental Safety*. **71**. 498 - 505.
- Tanabe, M., Szakonyi, G., Brown, K.A., Henderson, J.N. and Byrne, B. (2009) The multidrug resistance efflux complex, EmrAB from *Escherichia coli* forms a dimer invitro. *Biochemical and Biophysical Research*. **380**. 338 – 342.
- Tattaswart, U., Maillard, J-Y., Furr, J.R. and Russell, A.D. (1999) Comparative response of *Pseudomonas stutzeri* to antibacterial agents. *Journal of Applied Microbiology*. **87**. 323 – 331.
- Taylor, D.L. Bina, X.R. and Bina, J.E. (2012) *Vibrio cholerae* vexH encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the co-regulated pilus. *PloS ONE*. **7**. e38208.
- Thanabalu, T., Koronakis, E., Hughes, C. and Koronakis, V. (1998) Substrate-induced assembly of a contiguous channel for protein export from *E.coli*: reversible bridging of an inner membrane translocase to an outer membrane exit pore. *The EMBO Journal*. **17**. 6487 – 6496.
- The Merck Index (2006) An Encyclopedia of Chemicals, Drugs, and Biologicals. 14th Edition. Merck Inc., Whitehouse Station/Rahway, New Jersey,
- Thevenot, D., Dernburg, A. and Vernozy-Rozand, C. (2006) An updated review of *Listeria monocytogenes* in the pork meat industry and its products. *Journal of Applied Microbiology*. **101**. 7 –17.
- To, M.S., Favrin, S., Romanova, N. and Griffiths, M.W. (2002) Postadaptational resistance to benzalkonium chloride and subsequent physiochemical modifications of *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **68**. 5258 – 5264.
- Troller, J.A. (1993) *Sanitation in Food Processing, Second Edition (Food Science and Technology)*. Academic Press
- Tuncan, E.U. (1993). Effect of cold temperature on germicidal efficacy of quaternary ammonium compound iodophor and chlorine on *Listeria*. *Journal of Food Protection*. **56**. 1029 - 1033.
- Underwood, E. (2004) Good manufacturing practice. IN Fraiese, A.P., Lambert, P.A. and Maillard, J-Y (eds.) *Russell, Hugo and Aycliffe's Principles and Practice of Disinfection, Preservation and Sterilization*. Blackwell Publishing Ltd. pp 623 – 630.



Unnerstad, H., Bannerman, E., Bille, J., Danielsson, T.M.L., Waak, E. and Tham, W. (1996) Prolonged contamination of dairy with *Listeria monocytogenes*. *Netherlands Milk and Dairy Journal*. **50**. 493 – 499.

Vaara, M. and Vaara, T (1983) Polycations as outer membrane disorganizing agents. *Antimicrobial Agents and Chemotherapy*. **24**. 114 - 122.

Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiology Reviews*. **56**. 395 - 411.

Van Loosdrecht, M.C., Lyklema, J., Norde, W. and Zehnder, A.J.B. (1987) Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology*. **53**. 1898 – 1901.

Van Loosdrecht, M.C., Lyklema, J., Norde, W. and Zehnder, A.J.B. (1990) Influence of interfaces on microbial activity. *Microbiology Reviews*. **54**. 75 – 87.

Verran, J., Boyd, R.D. Hall, K., and West, R.H. (2001) Microbiological and chemical analysis of stainless steel and ceramics subjected to repeated soiling and cleaning treatments. *Journal of Food Protection*. **64**. 1377 – 1387.

Villalain, J., Mateo, C.R., Aranda, F.J., Shapiro, S. and Micol, V. (2001) Membranotropic effects of the antibacterial agent Triclosan. *Archives of Biochemistry and Biophysics*. **390**, 128 - 136.

Viveiros, M., Martins, A., Paixao, L., Rodrigues, L., Martins, M., Couto, I., Fahrnich, E., Kern, W.V. and Amaral, L. (2008) Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. *International Journal of Antimicrobial Agents*. **31**. 458 – 462.

Wainwright, M. (1998) Structure and biology of bacteria relevant to the action of disinfectants. IN Payne, K.R. (ed.) Critical Reports in Applied Chemistry. *Industrial Biocides*. Volume 23. John Wiley & Sons, pp2

Wexter, H. and Oppenheim, J.D. (1979) Isolation, characterization and biological properties of an endotoxin – like material from the Gram-positive organism *Listeria monocytogenes*. *Infection and Immunity*. **23**. 845 – 857.

Wirtanen, G. and Mattila – Sandholm, T. (1993) Epifluorescence image analysis and cultivation of foodborne biofilm bacteria grown on stainless steel surfaces. *Journal of Food Protection*. **56**, 678 – 683

World Health Organisation (WHO) (2012) Health topics; Foodborne Diseases [online]. [Accessed 23 March 2012]. Available at < [http://www.who.int/topics/foodborne\\_diseases/en/](http://www.who.int/topics/foodborne_diseases/en/)>

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